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Ph. D. Thesis – Short report

Expression of apoptosome pathway regulators and activation of apoptosome apparatus in non-small cell lung carcinoma

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SUMMARY

Background: The apoptosome pathway is interesting as potential therapeutic target because it plays an important role in the cancer chemotherapy- and biological therapy-induced apoptosis as well as in amplifying the death receptor and cytotoxic-granule-induced pathways. The functionality of apoptosome apparatus in non-small cell lung carcinoma (NSCLC) cells and tissues is often impaired due to defects in apoptosome pathway by changes in expression and/or acquired mutations and/or modifications of apoptosome components or its regulators that play a significant role in cancer cell proliferation and treatment unresponsiveness. Therefore, this thesis is aimed at the investigation of the expression status of apoptosome pathway regulators (survivin, HBXIP, XIAP, APIP, and UACA) and of the readiness of apoptosome apparatus activation in non-small cell lung carcinoma cells and tissues.

Methods: Following methods were used in this thesis: isolation and quantification of total RNA, real-time RT-PCR analysis, preparation of cell-free cytosol samples and extracts from cells and tissues, gel-filtration chromatography, Western blot analysis, enzyme analyses and cell culture techniques.

Results and conclusion: Non-small cell lung carcinoma has a higher predisposition to apoptosome-mediated apoptosis than normal lung tissue. The higher propensity of NSCLC tumours to apoptosome-mediated apoptosis is due to several factors. First, the increased expression of activatable apopotosome pathway core protein components, including Apaf-1 and procaspases-9 and -3. Second, the down-regulation of expression of *APIP* and *UACA* genes causing the lack of APIP-mediated apoptosome suppression and UACA-assisted Apaf-1 nuclear entry, which would lead to the failure of DNA damage checkpoint activation in NSCLC cells leading to their genomic instability and contributing to development and progression of NSCLC tumours. However, the functionality of apoptosome is suppressed in some NSCLC cell lines and in a high proportion of NSCLC tumours. There is evidence, that XIAP-mediated inhibition is not the major suppressor mechanism of apoptosome pathway induction in NSCLC tumours. Although overexpressed survivin in NSCLC together with abundant expression of HBXIP could lead to formation of the antiapoptotic survivin•HBXIP complex, which may be preferentially generated in lung tumours to inhibit the apoptosome pathway, in NSCLC cells, failure of the apoptosome-bound procaspase-9 activation may underlie the malfunction of apoptosome pathway.

SOUHRN

Úvod: Apoptosomový aparát má zásadní význam pro generování a amplifikaci apoptotických signálů a tím pro navození apoptosy v nádorových buňkách, přičemž účinnost protinádorové chemoterapie a radioterapie do značné míry závisí na spuštění apoptosy závislé na apoptosomu. Aktivace nebo funkce apoptosomového aparátu však často selhává. Příčinou mohou být změny v expresi a/nebo získané mutace a/nebo posttranslační úpravy komponentů apoptosomu či jeho regulátorů, které hrají významnou roli v proliferaci nádorových buněk a léčebné resistenci. Proto byla tato práce zaměřena zejména na analýzu exprese faktorů, které mohou regulovat spuštění a průběh apoptosomové kaspasové kaskády a na připravenost aktivace apoptosomového aparátu v buněčných liniích a tkáních nemalobuněčného karcinomu plic.

Metody: V této práci byly použity následující metody: izolace a kvantifikace celkové RNA, real-time RT-PCR, izolace bezbuněčného cytosolu a extraktů z buněk a tkání, gelová filtrace, Western blot analýza, enzymové analýzy a techniky kultivace buněčných linií.

Výsledky a závěry: Vyšší predispozice nemalobuněčného karcinomu plic k apoptosomem zprostředkované apoptose, v porovnání s normální plicní tkání, je způsobena několika faktory. Za prvé, zvýšenou expresí aktivovatelných apoptosomových komponentů v nádorů, včetně Apaf-1 a prokaspasy-9 a -3. Za druhé, sníženou expresí *APIP* a *UACA* genů způsobujících nedostatek APIP-zprostředkované inhibice apoptosomového aparátu a UACA-asistované Apaf-1 jaderné translokace, což by vedlo k selhání aktivace kontrolního bodu buněčného cyklu při poškození DNA vedoucí ke genomické nestabilitě a přispívání k rozvoji a progresi nádorů. Nicméně, v některých buněčných liniích a vysokém podílu nádorů nemalobuněčného karcinomu plic je funkce apoptosomového aparátu potlačena. Prokázalo se, že XIAP inhibitor se významně nepodílí na mechanismu suprese indukce apoptosomové dráhy v těchto nádorech. Ačkoliv zvýšená exprese survivinu v tkáních nemalobuněčného karcinomu plic spolu s dostatečnou expresí HBXIP proteinu může vést k formování antiapoptotického survivin•HBXIP komplexu, který je přednostně vytvořen v plicních nádorech a mohla by tak přispívat k potlačení apoptosomové dráhy apoptosy, zjistilo se, že v buňkách nemalobuněčného karcinomu plic je základem poruchy apoptosomové dráhy spíše selhání aktivace apoptosom-vázané prokaspasy-9.

CONTENT

1 INTRODUCTION

Lung cancer is statistically a leading cause of cancer-related death in Czech Republic and worldwide (Jemal *et al*, 2011; http://data.euro.who.int/dmdb, last accessed 13/9/2012). Incidence and mortality of lung cancer in Czech Republic is one of the highest in the world, slightly decreasing in men but increasing in women (http://www.svod.cz, last accessed 3/2/2013). Interestingly, more than 85% of all lung cancer patients are diagnosed with non-small cell lung cancer (NSCLC). Although a novel approach in the form of the so-called molecular targeted therapy is combined with the standard cytotoxic therapy to boost cisplatin/carboplatin effectiveness, only a modest progress in the 5-year-survival was achieved so far. Current treatment of NSCLC involves to large extent radiotherapy and/or a platinum-based chemotherapy, which primarily act, if efficacious, via inducing apoptotic cell death (Making and Dive, 2001; Reed, 2003; Liu *et al*, 2008a and b).

Apoptosis is characterized as a morphologically distinct type of cell death observed in various tissues and cells of multicellular organisms (Kerr *et al*, 1972; Galluzzi *et al*, 2007), by which an organism removes unwanted or harmful cells to maintain its homeostasis (Cecconi *et al*, 1998; Hao *et al*, 2005). The central role responsible for morphological changes typical for apoptosis is played by caspases, a family of cysteinyl aspartate-specific proteases (Fuentes-Prior and Salvesen, 2004). Signals to activate caspase zymogens involve extrinsic or intrinsic pathways, leading to cell devitalizing and dismantling by specific cleavage of plentiful cellular proteins (Fischer *et al*, 2003; Luthi and Martin, 2007; Timmer and Salvesen, 2007). Both the extrinsic and intrinsic pathways converge at or lead to mitochondrial-outer-membrane permeabilization (MOMP) and subsequent release of cytochrome-c (cyt-c) from mitochondria into the cytosol, which sets in motion the apoptosome pathway (Figure 1).

The apoptosome pathway is interesting as potential therapeutic target because it plays an important role in the cancer chemotherapy- and biological therapy-induced apoptosis as well as in amplifying the death receptor and cytotoxic-granule-induced pathways (Jiang and Wang, 2004; Liu *et al*, 2008). The functionality of apoptosome apparatus in NSCLC cells and tissues is often impaired play a significant role in cancer cell proliferation and treatment unresponsiveness (Krepela *et al*, 2004; Hoffarth *et al*, 2008). Therefore, in my thesis, I aimed at the investigation of the expression status of apoptosome pathway regulators (survivin, HBXIP, XIAP, APIP, and UACA) and of the readiness of apoptosome apparatus activation in non-small cell lung carcinoma (NSCLC) cells and tissues.

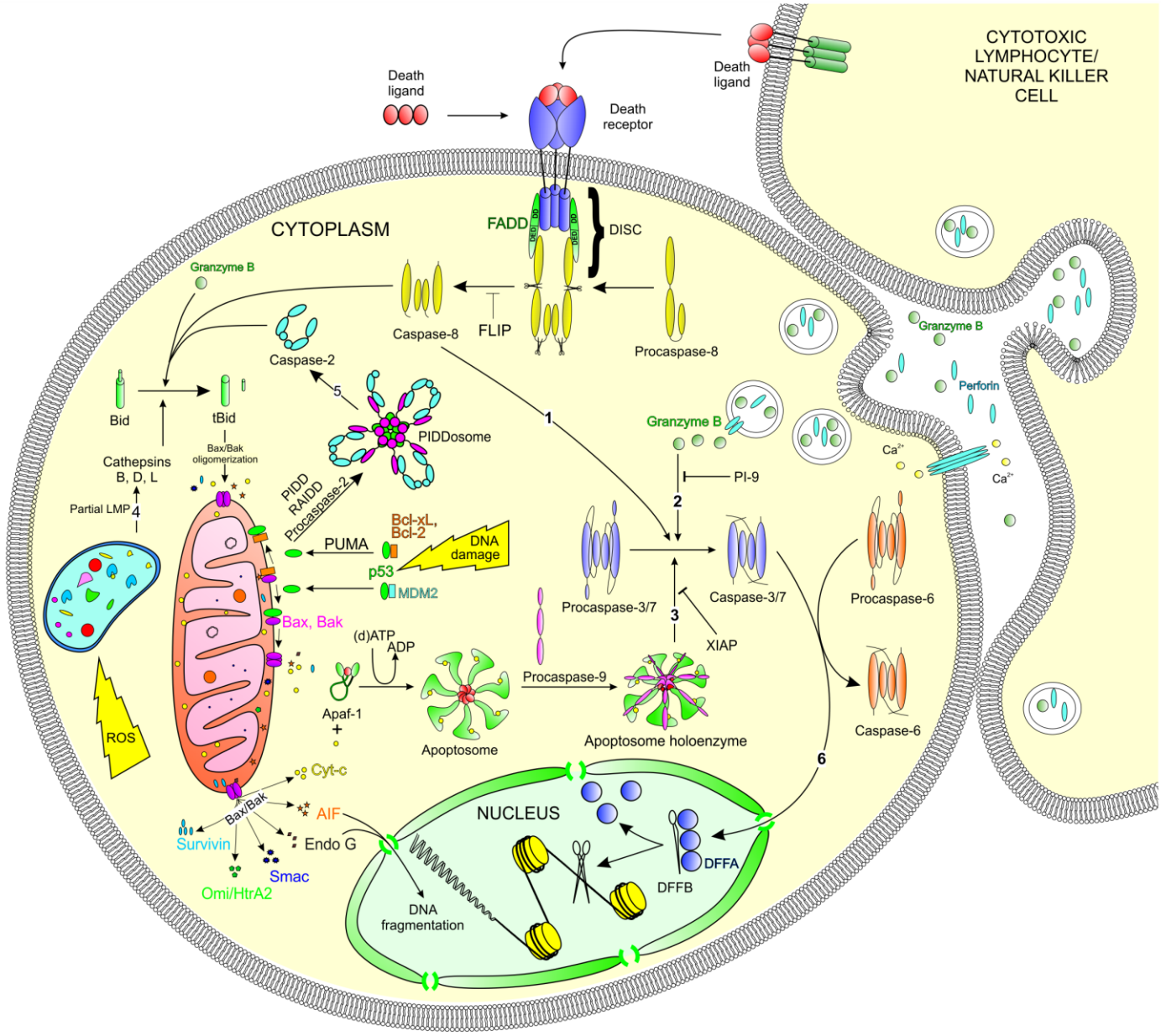

Figure 1. Caspase-dependent apoptotic pathways in cancer cells.
(1) Death ligand induces DISC formation, leading to procaspase-8 activation. Active caspase-8 proteolytically processes and activates executioner procaspase-3 and -7. This pathway can be inhibited by *e.g.* FLIP protein. (2) Cytotoxic lymphocyte or natural killer cell induce secretion of granzyme-B into the host cell, which in cytosol directly cleaves and activates procaspase-3 and -7. This pathway is antagonized by PI-9. [3] DNA damage induces MOMP via p53-mediated Bax/Bak oligomerization and release of antiapoptotic (Survivin) as well as proapoptotic factors (Omi/HtrA2, Smac, EndoG, AIF, cyt-c) from the mitochondrion into the cytosol. Cyt-c and (d)ATP induce Apaf-1 oligomerization following recruitment, processing and activation of procaspase-9. Caspase-9 in turn cleaves and thus activates procaspase-3 and -7. The pathway is suppressed by *e.g.* XIAP. (4) ROS induce partial LMP and release of cathepsins B, D and L, which cleave and thus activate Bid to MOMP-inducing tBid fragment. (5) Genotoxic stress leads to stabilization of p53 protein and p53-mediated expression of procaspase-2, which translocates from nucleus into the cytosol, where is activated in the PIDDosome. Active caspase-2 can induce MOMP by cleavage of Bid protein. (6) All pathways lead to activation of procaspase-3, -7, which when active, proteolytically cleave multiple proteins in the cytoplasm (e.g. procaspase-6, actin) or in the nucleus (e.g. DFFA subunit of DFF), which leading to DNA fragmentation, nuclear pyknosis, karryorhexis, cell shrinkage, plasma membrane blebbing and formation of apoptotic bodies, which are eliminated by phagocytes or neighbouring cells.

1.1 Mechanism of the apoptosome pathway

A classical mechanism to kill the cell by chemo- and radiotherapy is to cause irreparable DNA damage, and promote p53-mediated cell cycle arrest leading to apoptosis (Vazques *et al*, 2008). The key event in this pathway is the release of holocytochrome-c from the outer-membrane-permeabilized mitochondria through a Bax/Bak pore complex into the cytosol upon stress stimuli. Holocytochrome-c binds to apoptotic protease activating factor 1 (Apaf-1) to induce conformational changes in the presence of (d)ATP (Hu *et al*, 1999, Martin and Fearnhead, 2002). For full Apaf-1 activation, (d)ADP exchange for (d)ATP nucleotide is required (Li *et al*, 1997; Jiang and Wang, 2000). Subsequently, Apaf-1 activated monomers assemble into ~700 kDa heptameric circular complex called apoptosome (Acehan *et al*, 2002; Yu *et al*, 2005; Yuan *et al*, 2010).

The primary function of the apoptosome is the recruitment of procaspase-9 monomers via interaction of its CARD with the Apaf-1 CARDs. Two procaspase-9 activation models have been proposed. In the proximity induced dimerization model, apoptosome facilitates the dimerization of procaspases-9 molecules (Boatright *et al*, 2003; Pop *et al*, 2006). However, within homodimers of caspase-9 molecules, which are active, only one caspase-9 molecule contains the catalytically competent active site (Renatus *et al*, 2001). Moreover, engineered caspase-9 molecules, that form constitutive dimers outside the apoptosome, are much less active than the caspase-9 activated in the apoptosome-dependent manner (Chao *et al*, 2005). In the allosteric model, the binding of procaspase-9 monomer to apoptosome induces a conformational change in the bound procaspase-9 molecule (Rodriguez and Lazebnik, 1999; Shiozaki *et al*, 2002; Chao *et al*, 2005). Furthermore, no proteolytic cleavage is needed for procaspase-9 activation (Rodriguez and Lazebnik, 1999; Renatus *et al*, 2001; Boatright *et al*, 2003). These data together with the recent biochemical (Malladi *et al*., 2009) and structural data (Yuan *et al*., 2011) strongly support the apoptosome-dependent allosteric mechanism of procaspase-9 activation. The apoptosome-activated and -associated procaspase-9 molecule mediates by itself its own proteolytic processing. As a result, two caspase-9 subunits are generated, the large p35 one and the small p12 one (Yin *et al*, 2006). This cleavage not only lowers the K_M of caspase-9 for procaspase-3 substrate (Zou *et al*, 2003), but also it activates a molecular cycle, in which the apoptosome bound caspase-9 is replaced by procaspase-9 molecules with higher affinity to the apoptosome (Malladi *et al*, 2009). The purpose of this binding-processing-replacement cycle is to activate procaspase-9 monomers to caspase-9, which only in the association with apoptosome is able to proteolytically process and activate

procaspase-3 (Malladi *et al*, 2009). The substrates for the apoptosome-associated caspase-9 are the effector procaspase-3 and -7.(Srinivasula *et al*., 1998; Yin *et al*., 2006; Twiddy *et al*, 2006; Malladi *et al*, 2009; Yuan *et al*, 2011).

1.2 Regulation of the apoptosome pathway in cancer

Under pathological conditions, a robust induction of apoptosome pathway is essential to kill the cells, otherwise, the apoptotic signaling is vanquished in the presence of a variety of mechanisms that protect the cell from unwanted apoptosis (Schafer and Kornbluth, 2006; Bratton and Salvesen, 2010; Florentin and Arama, 2012). Moreover, cell's propensity to apoptosis is set by the level of the key AA components expressed by a cell.

Regulation of expression of the key apoptosome apparatus components

DNA methylation, as an epigenetic regulation mechanism, might be involved in downregulation of apoptosis-associated and tumour suppressor genes in cancer cells with overexpressed DNA methyltransferases as a consequence of carcinogenesis. This could be the case of low level of Apaf-1 in some cancer cells, as the DNA methylation inhibitor 5-aza-2'-deoxycytidine can restore the physiological level of Apaf-1 and increase the sensitivity to apoptosis in melanoma and leukemic cells (Soengas *et al*, 2001; Fu *et al*, 2003; Furukawa *et al*, 2005). Moreover, it was demonstrated that increased expression of caspase-9 could be achieved either by DNA demethylating agent decitabine or E2F-1 transcriptional factor, and that treatments with these compound can sensitise lung cancer cells to chemotherapy inducing apoptosis (Nahle *et al*, 2002; Gomyo *et al*, 2004).

Increasing evidence shows that alternative splicing of certain pre-mRNAs has functional importance in cancer development. Five splice variants of Apaf-1 were found expressed in cancer cells: Apaf-1XL, Apaf-1LN, Apaf-1LC, Apaf-1S and Apaf-1ALT. However, only the isoforms containing 13 WD-40 repeats (Apaf-1XL and Apaf-1LC) are activatable by cyt-c to induce apoptosis (Benedict *et al*, 2000; Ogawa *et al*, 2003). Importantly, Apaf-1ALT is able to negatively interfere with Apaf-1XL function and sequester procaspase-9 by CARD interactions and thus could slower the apoptosis. (Ogawa *et al*, 2003).

To date, three splice variants of human procaspase-9 have been identified, caspase-9-α (Duan *et al*, 1996, Srinivasula *et al*, 1996), caspase-9-β/S/b (Izawa *et al,* 1999; Seol and Billiar, 1999; Srinivasula *et al*, 1999) and caspase-9-γ (Wang *et al*, 2006). Only the full length caspase-9-α retains the catalytic activity. Caspase-9-β isoform is missing most of the central large subunit

domain including the catalytic site, while caspase-9-γ lacks both, the large and the small subunits domains, therefore, procaspase-9-β and -γ can compete with full length procaspase-9-α for binding to Apaf-1 CARD, thus inhibiting apoptosis (Seol and Billiar, 1999; Srinivasula *et al*, 1999; Wang *et al*, 2006).

Posttranslational regulation of apoptosome apparatus assembly and function

Protein phosphorylation is a common mechanism for posttranslational regulation of variety of apoptotic but also non-apoptotic factors. On one hand, phosphorylation of a particular protein can activate, or, on the other hand, inhibit its function. Both core apoptosome components were shown to undergo this modification and if phosphorylated at specific site, the activity of AA component is either suppressed, or activated (Allan and Clare, 2009; Kim *et al*, 2012).

Except phosphorylation, proteolytic modification of AA components was shown to affect their function. Once the apoptosome-associated caspase-9 p35/p12 form proteolytically converts procaspase-3 to active caspase-3, the active caspase-3 can cleave both the procaspase-9 monomer at D330 and the N-terminus of the caspase-9 small subunit p12 at D330 as well, thereby removing the intersubunit linker peptide, which results in generation of caspase-9 p37/p10 and caspase-9 p35/p10 forms, respectively (Zou *et al*, 2003). This cleavage removes the ATPF sequence motif from the N-terminus of the caspase-9 small subunit p12 that is needed for caspase-9 inhibition by XIAP (Zou *et al*, 2003; Denault *et al*, 2007). Furthermore, it was shown, that Apaf-1 monomer is also the proteolytic target of the active caspase-3 (Bratton *et al*, 2001, Lauber *et al*, 2001). Apaf-1 monomer is cleaved by active caspase-3 resulting in partial removal of the CARD and WD-40 repeats. The resulted 84 kDa Apaf-1 is able to oligomerize into a 400 kDa Apaf-1 heptamer in the absence of cyt-c but cannot associate with procaspase-9. This feedback mechanism of degradation of Apaf-1 may decrease or cease the propagation of the death signal in cancer cells (Lauber *et al*, 2001). Moreover, Apaf-1 can also be cleaved into the resulting p30 fragment, which can interact with the proteolytically inactive 1.4 MDa apoptosome (Bratton *et al*, 2001).

APIP

The Apaf-1-interacting protein (APIP) is an endogenous competitive inhibitor of apoptosome-mediated procaspase-9 activation (Cao *et al*, 2004, Cho *et al*, 2004). It was found, that under ischemic condition, APIP is highly expressed in skeletal muscle, heart and neurons and prevents them from ischemic damage by caspase-9-mediated apoptosis inhibition (Cao *et al*, 2004, Cho *et al*, 2004). NSCLC tumour microenvironment also contains ischemic regions

characterized by hypoxia and acidosis (Graves *et al*, 2010). Therefore, APIP might play a role in suppression of apoptosome pathway in NSCLC tumours.

APIP negatively competes with procaspase-9 recruitment to AA upon apoptotic stimuli (Cao *et al*, 2004). Moreover, under ischemic conditions, APIP induces and sustains the activation of Akt and ERK1/2 kinases, which phosphorylate procaspase-9, thus inhibiting its AA-mediated activation (Cho *et al*, 2007). Recently, APIP was shown to be identical with 5'-methylthioribulose-1-phosphate dehydratase (EC: 4.2.1.109) (Mary *et al*, 2012), which reduced expression was associated with increased sensitivity to the chemotherapeutic agent carboplatin (Ko *et al*, 2012).

UACA

Uveal autoantigen with coiled-coil domains and ankyrin repeats (UACA) (Yamada *et al*, 2001) or nucling (Sakai *et al*, 2003) is a proapoptotic factor that plays a role in the regulation of proapoptotic and nuclear factor-κB (NF-κB) signaling (Sakai *et al*, 2003; Sakai *et al*, 2004; Liu *et al*, 2009). Proapoptotic stimuli such as treatment with adriamycin or heat shock upregulate nucling expression (Sakai *et al*, 2004). It was shown, that nucling stabilizes the apoptosome complex components *in vivo* and promotes caspase-dependent cell death (Sakai *et al*, 2004). Nucling induces Apaf-1 expression and its translocation into the nucleus (Sakai *et al*, 2004). Moreover, nucling interaction with NF-κB p50 and p65 subunits prevents its nuclear translocation and thus reduces expression of NF-κB-targeted genes, which products might support cell survival (Liu *et al*, 2004; Liu *et al*, 2009).

Inhibitor of apoptosis proteins

The most studied IAPs are XIAP, for its ability to inhibit caspase activity directly (Eckelman *et al*, 2006), and survivin, which is currently studied as a therapeutic target due to its increased expression in the vast majority of cancers over normal cells (Duffy *et al*, 2007; Altieri, 2012; Arora *et al*, 2012; Chen *et al*, 2012; Khan *et al*, 2012). The overexpression of both, XIAP and survivin, in some cancer cells is linked with the apoptosis suppression in cancer cells (Dohi *et al*, 2004; Galban *et al*, 2009; Chen *et al*, 2012) and as well as with chemotherapy- and radiation-induced apoptosis resistance in NSCLC (Hu *et al*, 2003; Cao *et al*, 2004; Lu *et al*, 2004; Dong *et al*, 2006).

These antiapoptotic functions of XIAP and survivin suggest that they might contribute to tumourigenesis and NSCLC progression.

2 AIMS OF THE THESIS

Although non-small cell lung carcinoma (NSCLC) cells express the key cytosolic components of apoptosome apparatus (AA) (Apaf-1, procaspase-9) and its main effector procaspase-3, the activatability or the functionality of the apoptosome apparatus often fails. To date, numerous negative regulators of the assembly or the function of the apoptosome have been described. However, the mechanism of endogenous dysfunction of AA in non-small cell lung carcinoma still remains elusive.

The aims of the thesis were to analyse the expression and activity of molecular factors that might regulate the initiation or the course of the apoptosome cascade in NSCLC cells and tissues of various histopathological types.

The specific aims of this thesis were:

- Analysis of expression status of survivin, HBXIP, APIP - factors blocking the apoptosome assembly, in NSCLC tissues and cells
- Analysis of expression status of XIAP - factor inhibiting the proteolytic activity of the apoptosome apparatus, in NSCLC tissues and cells
- Analysis of expression status of nucling/UACA - factor responsible for translocation of AA and Apaf-1 into the nucleus, in NSCLC tissues and cells
- Analysis of DNA methylation involvement in the expression of Apaf-1, procaspase-9 and procaspase -3 mRNAs in NSCLC cells
- Analysis of expression status of Apaf-1 mRNA variants -XL/-LC and -LN/-S in NSCLC tumours and lungs
- Analysis of expression status of procaspase-9 and caspase-9S mRNAs in NSCLC tumours and lungs
- Analysis of apoptosome functionality in NSCLC tissues and cells
- Analysis of apoptosome assembly in NSCLC cells

3 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, preparation of cell-free cytosol samples and extracts from cells and tissues, gel-filtration chromatography, Western blot analysis, enzyme analyses and cell culture techniques, are described in the publications related to the thesis (c.f. section List of publications).

4 RESULTS

4.1. Expression status of survivin, HBXIP, XIAP, APIP and UACA

To analyse the expression status of apoptosome apparatus regulators, survivin, HBXIP, XIAP, APIP and UACA mRNAs in NSCLC tumours and lungs, we quantitated the level of these transcripts and of an endogenous reference transcript, β-actin mRNA, by means of real-time RT-PCR. Survivin and XIAP genes were strongly expressed in the studied NSCLC cell lines at both the mRNA and protein levels (Fig. 2).

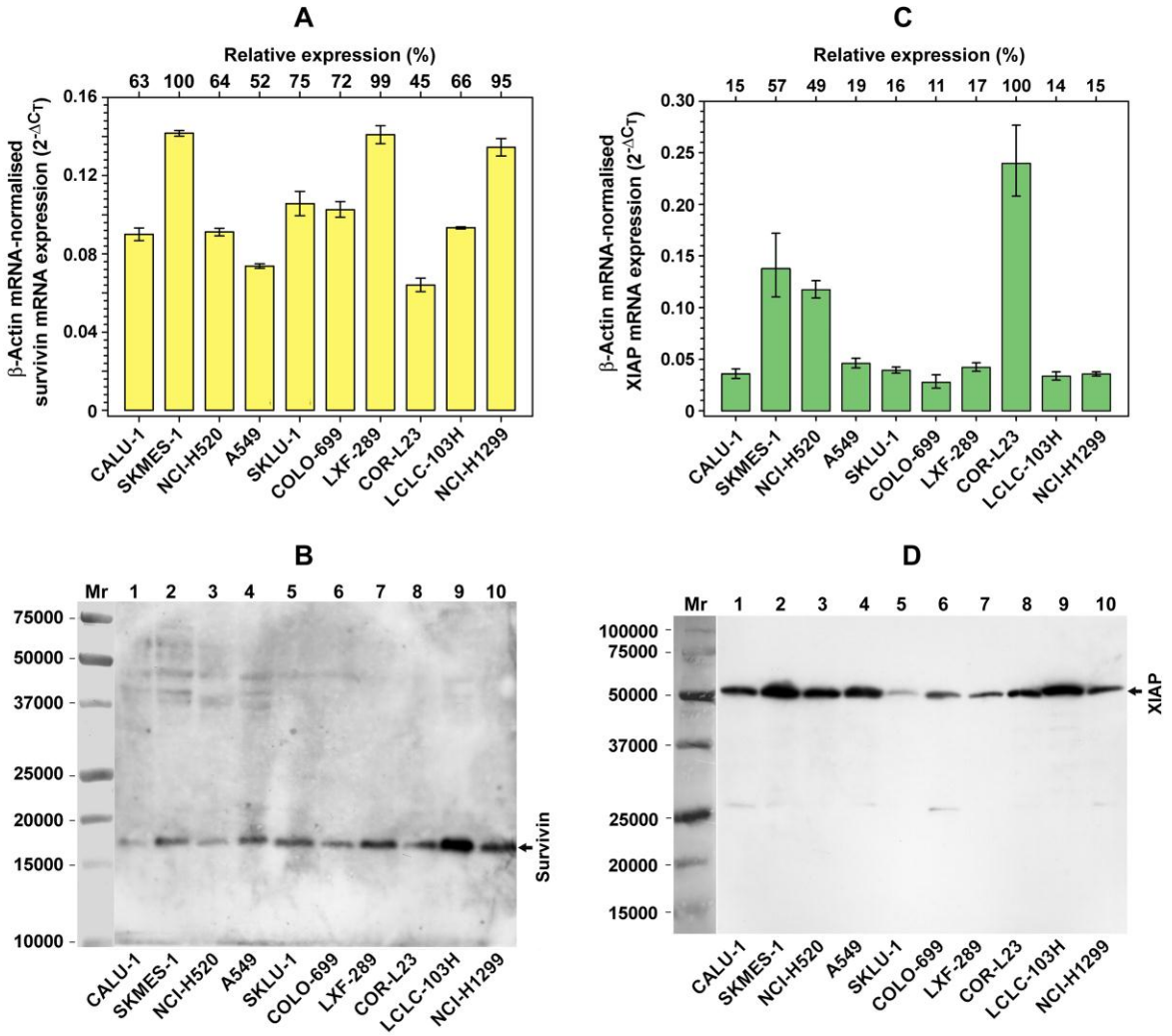

Figure 2. Analysis of survivin and XIAP expression in non-small cell lung carcinoma cell lines.
(A and C) Expression of survivin and XIAP mRNAs in the tumour cell lines as quantitated by real time RT-PCR. The data are indicated as mean ± standard error of the mean of three independent experiments. (B and D) Expression of survivin and XIAP proteins in the tumour cell lines as analysed by SDS-PAGE and immunoblotting-ECL

The expression of survivin protein was highly upregulated also in NSCLC tumours as compared to matched lungs (Table I). More than tenfold higher level of survivin protein was observed in 72 (82%) of 88 examined NSCLC tumours as compared to the lungs.

Regarding XIAP expression, there was significantly higher expression of XIAP protein in NSCLC tumours, and particularly in LAC tumours, as compared to matched lungs (Table II).

Table I. Immunometric analysis of survivin protein expression in NSCLC tumours and lungs.

Tumour type	n	Survivin protein expression (ng/mg of total protein) [b]		Statistical difference (*P*) of survivin protein expression in Tu *versus* Lu [c]	Tu/Lu ratio of survivin protein expression [b]	Number of patients with Tu/Lu survivin protein expression ratio ≥ 2 and ≤ 0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC	88 [a]	2.383 (0.107 – 15.901)	0.089 (0.010 – 0.501)	4.7×10^{-29}	23.5 (1.1 – 720)	85 (97%) and 0
SQCLC	39	2.148 (0.107 – 15.901)	0.083 (0.015 – 0.325)	3.2×10^{-13}	27.2 (1.1 – 166.2)	37 (95%) and 0
LAC	38	2.189 (0.224 – 11.365)	0.079 (0.014 – 0.469)	7.8×10^{-14}	24.0 (1.7 – 142.7)	37 (97%) and 0

[a] A total of 88 NSCLC patients was studied including 39 patients with SQCLC, 38 patients with LAC, 3 patients with SQCLC+LAC mixed type tumour, 2 patients with LCLC, 3 patients with SLC, and 3 patients with UNDIF.
[b] Data indicated as median with the range in parentheses.
[c] Statistical difference of survivin protein expression in Tu *versus* Lu was calculated by Mann-Whitney test.

Table II. Immunometric analysis of XIAP protein expression in NSCLC tumours and lungs.

Tumour type	n	XIAP protein expression (ng/mg of total protein) [b]		Statistical difference (*P*) of XIAP protein expression in Tu *versus* Lu [c]	Tu/Lu ratio of XIAP protein expression [b]	Number of patients with Tu/Lu XIAP protein expression ratio ≥ 2 and ≤ 0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC	88 [a]	34.01 (0.24 – 112.70)	29.93 (3.40 – 76.40)	0.017	1.3 (0.01 – 8.3)	14 (16%) and 12 (14%)
SQCLC	39	32.86 (0.24 – 86.27)	30.07 (3.40 – 69.46)	0.280	1.1 (0.01 – 8.1)	4 (10%) and 5 (13%)
LAC	38	41.47 (0.73 – 112.70)	27.73 (14.05 – 76.41)	0.004	1.4 (0.03 – 5.4)	8 (21%) and 4 (11)

[a] A total of 88 NSCLC patients was studied including 39 patients with SQCLC, 38 patients with LAC, 3 patients with SQCLC+LAC mixed type tumours, 2 patients with LCLC, 3 patients with SLC and 3 patients with UNDIF.
[b] Data indicated as median with the range in parentheses.
[c] Statistical difference of XIAP protein expression in Tu *versus* Lu was calculated by Mann-Whitney test.

The analyses of APIP and UACA mRNAs and proteins expression revealed, that *APIP* and *UACA* genes were expressed in all tested NSCLC cell lines on the level of both mRNA and protein, but the levels of their expression were quite variable (Figure 3). Moreover, APIP and UACA mRNAs and proteins expressions were downregulation in NSCLC tumours including the major histopathological types of NSCLC (SQCLC and LAC) as compared to matched lungs (Figure 4). Although the tumour stage did not have significant impact on the expression of APIP mRNA in NSCLC tumours, the expression of UACA mRNA was significantly lower in stage IA tumours as compared to stage IB tumours and higher stage tumours. Moreover, the down-regulation of UACA mRNA expression was relatively more frequent in stage IA tumours (in 14 of 15 patients, 93%) than in stage IB tumours (in 29 of 42 patients, 69%) and stage II + III tumours (in 28 of 41 patients, 68%). Patient's gender, smoking status and tumour grade did not significantly affect the expression neither of APIP nor UACA mRNAs

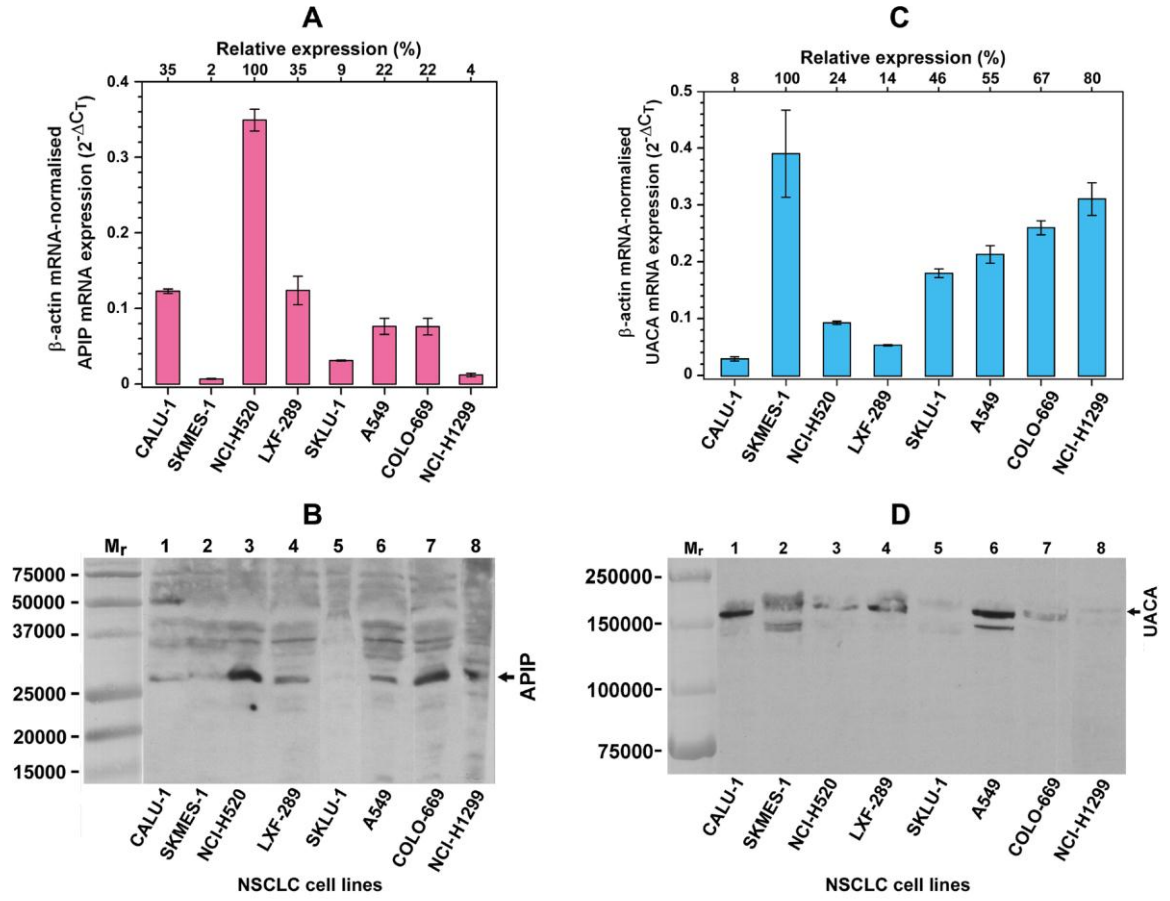

Figure 3. Analysis of APIP and UACA expression in non-small cell lung carcinoma cell lines.
(A and C) Expression of APIP and UACA mRNAs in the tumour cell lines as quantitated by real time RT-PCR. The data are indicated as mean ± standard error of the mean of three independent experiments. (B and D) Expression of APIP and UACA proteins in the tumour cell lines as analysed by SDS-PAGE and immunoblotting-ECL.

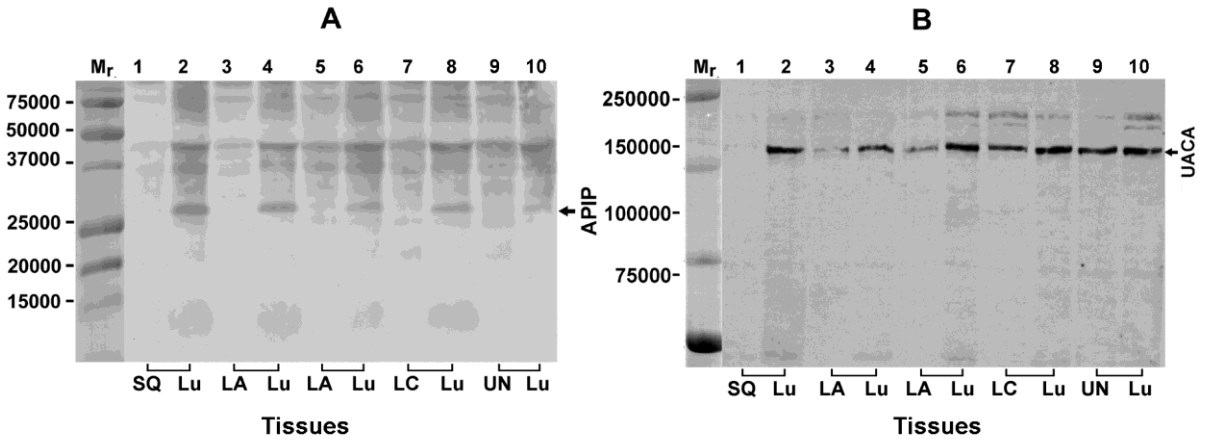

Figure 4. Analysis of APIP and UACA protein expression in non-small cell lung carcinoma tissues.
Expression of (A) APIP and (B) UACA proteins in non-small cell lung carcinoma tissues as analysed by SDS-PAGE and immunoblotting-ECL.

Expression status of Apaf-1 and caspase-9 mRNA splice variants in NSCLC tumours and lungs

We further investigated the expression status of Apaf-1 mRNA variants -XL/-LC and –LN/-S, encoding the (cyt-c+dATP)-activatable and the (cyt-c+dATP)-non-activatable Apaf-1 protein isoforms, respectively, in 50 NSCLC tumours of 5 histopathological types and matched lung parenchyma from surgically treated lung cancer patients. We found that the expression of Apaf1-XL/-LC mRNAs was significantly higher compared to the expression of Apaf-1-LN/-S mRNAs in NSCLC tumours ($P = 1.7 \times 10^{-14}$; Mann-Whitney test) (Figure 5A).

We also investigated the expression levels of procaspase-9 and caspase-9S mRNAs in NSCLC tumours and matched lung parenchyma. Uncoupled real-time RT-PCR analysis showed that the expression of procaspase-9 mRNA was significantly higher compared to caspase-9S mRNA in 63 NSCLC tumours ($P = 4.2 \times 10^{-17}$; Mann-Whitney test) as well as in matched lungs ($P = 6.0 \times 10^{-20}$; Mann-Whitney test) (Figure 5B).

Activatability of apoptosome apparatus in NSCLC cell lines

To assess the functionality of apoptosome apparatus (AA) in NSCLC cell lines, we studied its activatability by monitoring the generation of caspase-3-like activity upon preincubation of cell-free cytosols with cyt-c and dATP. The (cyt-c + dATP)-dependent caspase-3-like activity was induced in 5 (CALU-1, NCI-H520, SKMES-1, LXF-289 and COLO-699) of 7 studied NSCLC cell lines (Figure 6). The samples of cell-free cytosol from A549 and SKLU-1 cell lines were resistant to the (cyt-c+dATP)-mediated induction of caspase-3-like activity.

Interestingly, a high level of caspase-3-like activity was already present in the (cyt-c + dATP)-untreated cell-free cytosol from SKLU-1 cells (Figure 6). Both, the endogenous and

the (cyt-c + dATP)-induced caspase-3-like activities were completely inhibited by the caspase inhibitor Ac-DEVD-CHO at 10 μM (data not shown).

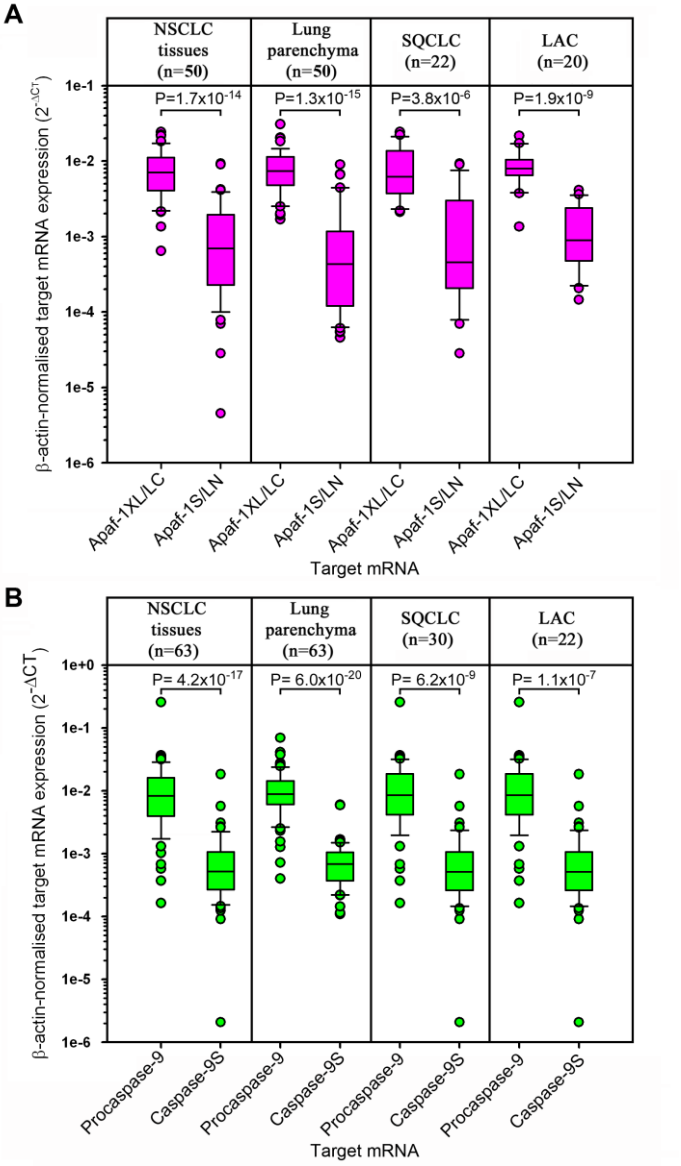

Figure 5. Expression of Apaf-1 and procaspase-9 mRNA variants in NSCLC tissues and lungs.
The expression of (A) Apaf-1XL/-LC and Apaf-1LN/-S mRNA variants and (B) procaspase-9 and caspase-9S mRNA variants was quantitated by uncoupled real-time RT-PCR assays, using an input of 200 ng of total RNA, and the expression data were normalized against the expression of β-actin mRNA (an internal reference transcript). The expression data are shown as median (the horizontal line inside the box) with the upper ranges of 75% and 90% and the lower ranges of 25% and 10%. Statistical differences were calculated by Mann-Whitney test.

Apoptosome complexes assembly in cell-free cytosol from NSCLC cell lines

To gain an insight into apoptosome dysfunction, we analysed the capability of Apaf-1 and procaspase-9 to assemble into apoptosome complexes using gel filtration chromatography (GFC) followed by SDS-PAGE and immunoblotting-ECL. As a unincubated control, we used cell-free cytosol from COLO-669 cells which did not receive exogenous cyt-c and dATP and was not incubated prior to GFC. Using this material, Apaf-1 and procaspase-9 eluted in a M_r-region corresponding to their unoligomerized forms (Figure 7A). Surprisingly, if cell-free cytosol from COLO-669 cells was incubated alone at 37˚C for 30 min, procaspase-9 eluted

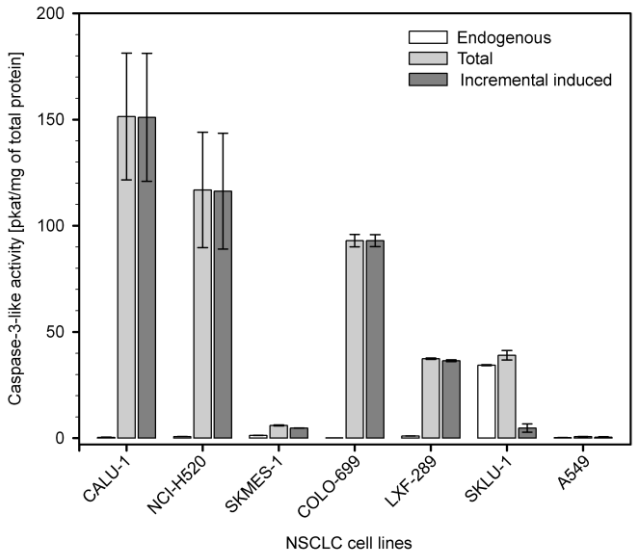

Figure 6. Analysis of the endogenous and the (cyt-c+dATP)-induced caspase-3-like activity in cell-free cytosol samples from NSCLC cells. Cell-free cytosol samples were preincubated without (endogenous caspase-3-like activity) or with 10 μM cyt-c and 1 mM dATP (total caspase-3-like activity) at 37˚C for 30 min (2.5 mg of total cytosolic protein /ml). Samples of the preincubated cytosol were then added into the reaction with Ac-DEVD-AFC as substrate and the fluorescence of enzymatically released AFC was measured. The incremental-induced caspase-3-like activity was calculated by subtracting the endogenous caspase-3-like activity from the total caspase-3-like activity.

in the same M_r-region as in non-incubated control, but Apaf-1 eluted in two M_r-regions, one corresponding to its high-M_r complexes or aggregates and the other one to its unoligomerized forms (Figure 7B).

By contrast, if the cell-free cytosol from COLO-669 cells was incubated with cyt-c and dATP at 37˚C for 30 min, procaspase-9 was completely converted to caspase-9, which coeluted with Apaf-1 in two M_r-regions, one corresponding to their high-M_r complexes and the other one to their unoligomerized forms (Figure 7C).

On the other hand, the incubation of A549 cell-free cytosol with cyt-c and dATP induced a shift of a portion of both Apaf-1 and procaspase-9 proteins to high-M_r fractions. These fraction coeluted and contained predominantly procaspase-9 while the caspase-9 p35 and p37 forms were detectable in the high-M_r fractions only in trace amounts. However, the majority of procaspase-9 and some caspase-9 eluted in the low-M_r fractions (Figure 8).

Apoptosome-dependent procaspase-9 proteolytic processing and caspase-9 activity

In cell-free cytosols from CALU-1 and SKMES-1 cells, we detected the generation of the p35 caspase-9 subunit already within 5 min after the addition of cyt-c and dATP into cell-free cytosol, while the p37 caspase-9 subunit appeared later, usually in 10 min after the addition of cyt-c and dATP into cell-free cytosol (Figure 9A). Surprisingly, in the (cyt-c+dATP)-activated cell-free cytosol from COLO-699 and LXF-289 cells the p35 caspase-9 subunit, detected in 10 min of activation, became undetectable later (Figure 9B). In cell-free cytosol

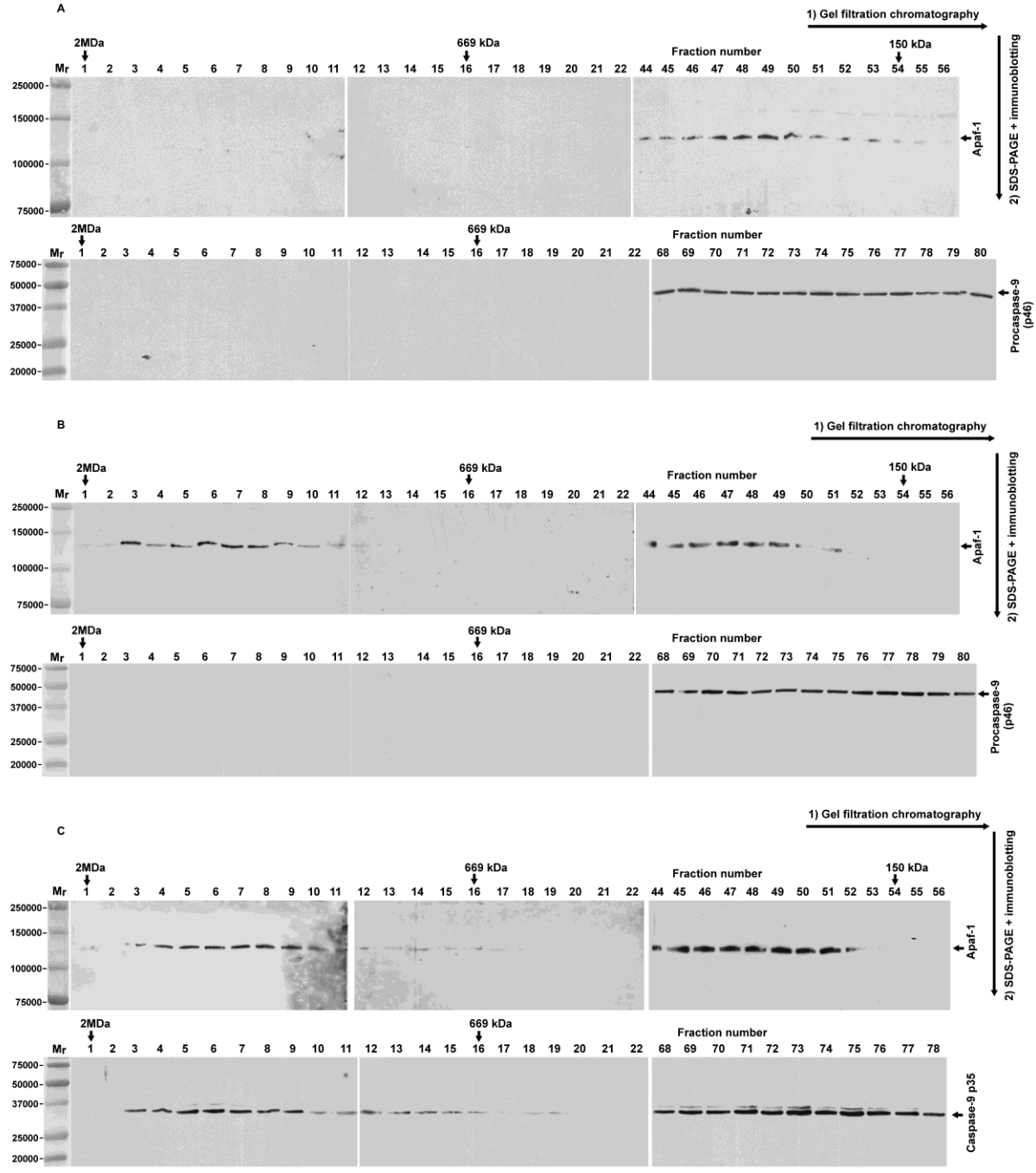

Figure 7. Analysis of apoptosome complexes formation in COLO-699 cell-free cytosol.

COLO-699 cell-free cytosol (5 mg of total protein/ml) was either kept unincubated at 0 - 4 ^{o}C or it was incubated at 37˚C for 30 min in the absence or the presence of exogenous cyt-c (10μM) + dATP (1mM) prior to its separation by gel filtration chromatography on a column of Sephacryl S300HR. Selected fractions were subjected to analysis by SDS-PAGE and immunoblotting-ECL as described in Materials and Methods.
(A) Unincubated control cell-free cytosol: Apaf-1 and unprocessed procaspase-9 eluted in low M_r-fractions.
(B) Cell-free cytosol after incubation without of exogenous cyt-c and dATP: Apaf-1 eluted in both high and low M_r-fractions, while procaspase-9 eluted in low M_r-fractions only.
(C) Cell-free cytosol after incubation with exogenous cyt-c and dATP: Apaf-1 eluted in both high and low M_r-fractions, while procaspase-9 was completely converted to caspase-9 p35 form which coeluted with Apaf-1 in high M_r-fractions and also eluted in low M_r-fractions. The positions of M_r markers are indicated with arrows.

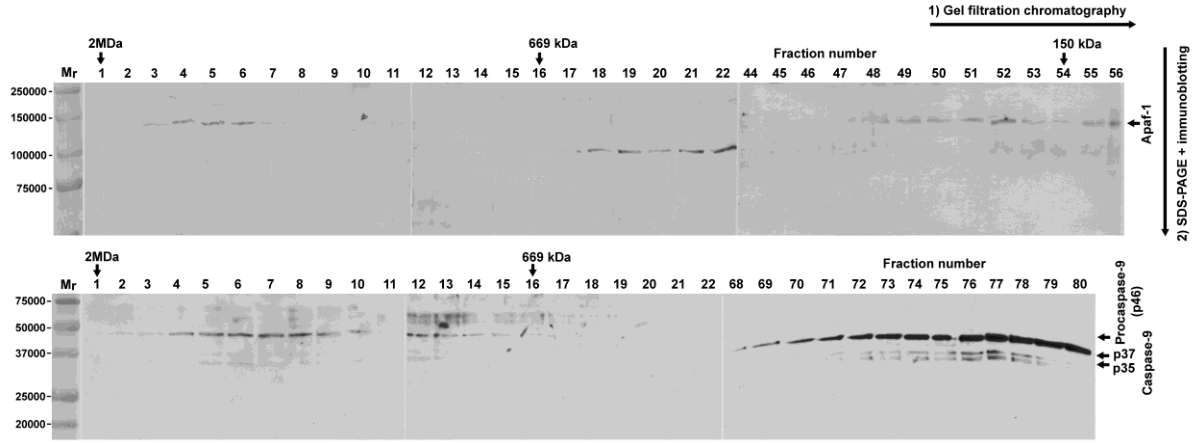

Figure 8. Analysis of apoptosome complexes formation in A549 cell-free cytosol.

A549 cell-free cytosol was incubated with cyt-c and dATP at 37°C for 30 min. The sample (5 mg of total protein/ml) was fractionated on a column of Sephacryl S300HR and selected fractions were subjected to SDS-PAGE and immunoblotting-ECL analysis as described in Materials and Methods. Apaf-1 and procaspase-9 eluted in high-M_r fractions as well as low-M_r fractions. Trace amounts of p35 and p37 forms of caspase-9 were found in low-molecular fractions. The positions of Mr markers are indicated with arrows.

from A549 incubated with cyt-c and dATP, the generation of p35 and p37 caspase-9 subunits was not detected and only a protein band corresponding to proscaspase-9 (p46) was found (Figure 9C). Finally, in SKLU-1 cell-free cytosol, which was proved to exhibit a significant endogenous level of caspase-3-like activity (Figure 9), the p35 caspase-9 subunit was not generated by exogenously added cyt-c and dATP, while the p37 caspase-9 subunit was detected in both the control and the (cyt-c+dATP)-treated samples (Figure 9). This indicates that procaspase-9 from SKLU-1 cells is resistant to the apoptosome-dependent activation and that the caspase-3-like enzyme in viable SKLU-1 cells may be involved in extra-apoptosome procaspase-9 processing. To study further the procaspase-9 processing, we incubated cell-free cytosol from NCI-H520 cells for different times with cyt-c and dATP and in the absence or the presence of the caspase inhibitor Ac-DEVD-CHO. The Western blot analysis showed that in the absence of the caspase inhibitor, procaspase-9 was processed into two caspase-9 forms, one containing the p35 subunit and other one containing the p37 subunit (Fig. 10A, left panel). The formation of the p35 caspase-9 form preceded the generation of the p37 caspase-9 form (Fig. 10A, lanes 1 and 2). The time-dependent loss of procaspase-9 was faster in samples without Ac-DEVD-CHO compared to the samples with Ac-DEVD-CHO (Figure 10A, the left and right panel). In addition, the formation of the caspase-9 p37 form correlated with the generation of the p20 and p17 forms of the large subunit of active caspase-3 (Figure 10A and 10B, left panels), since both the processing of procaspase-9 to the p37 form of caspase-9 and the generation of active caspase-3 p20/p17 forms were completely blocked in

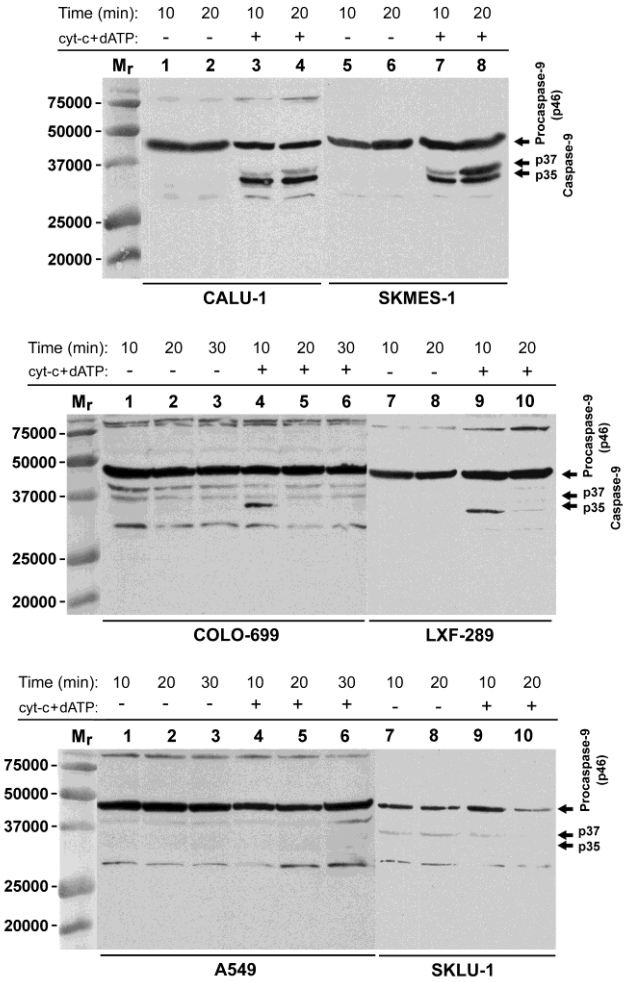

Figure 9. Analysis of procaspase-9 processing in cell-free cytosols from NSCLC cell lines.

Aliquots of cytosols from (A) CALU-1 and SKMES-1 (B) COLO-699 and LXF-289 (C) A549 and SKLU-1 cells were preincubated with or without cyt-c and dATP at 37˚C for indicated times. The reactions were stopped on ice by adding the SDS-PAGE sample buffer. After denaturation, the samples were subjected to SDS-PAGE and were immunoblotted with an anti-caspase-9 antibody. The arrows indicate the full length procaspase-9 precursor (p46) and the subunits p35 and p37 of two caspase-9 forms resulting from the proteolytic processing of procaspase-9.

the presence of Ac-DEVD-CHO (Figure 10A and B, right panels). These results indicate that Ac-DEVD-CHO, a typical caspase-3 inhibitor, does not inhibit the apoptosome-dependent procaspase-9 activity, which manifests itself by the autoproteolytic formation of caspase-9 p35 form, but it does inhibit the active, *i.e.* the apoptosome associated, caspase-9 p35 form, which initiates the proteolytic activation of procaspase-3. The result of these experiments shed light on the target of the Ac-DEVD-CHO- mediated inhibition of the (cyt-c + dATP)-induced caspase-3-like activity, we observed in control reaction done in studies of apoptosome activation (data not shown). During these control enzyme reactions, Ac-DEVD-CHO (10 μM) inhibited the activity of the apoptosome-associated caspase-9 but not the activity of caspase-3/-7, because the later enzymes could not be generated due to preceding caspase-9 inhibition.

Thermal effect on the (cyt-c + dATP)- induced apoptosome apparatus activation

To asses the effect of the thermal pretreatment on the (cyt-c + dATP)-induced caspase-3-like activity, we preincubated cell-free cytosol from NCI-H520 cells without cyt-c and dATP at

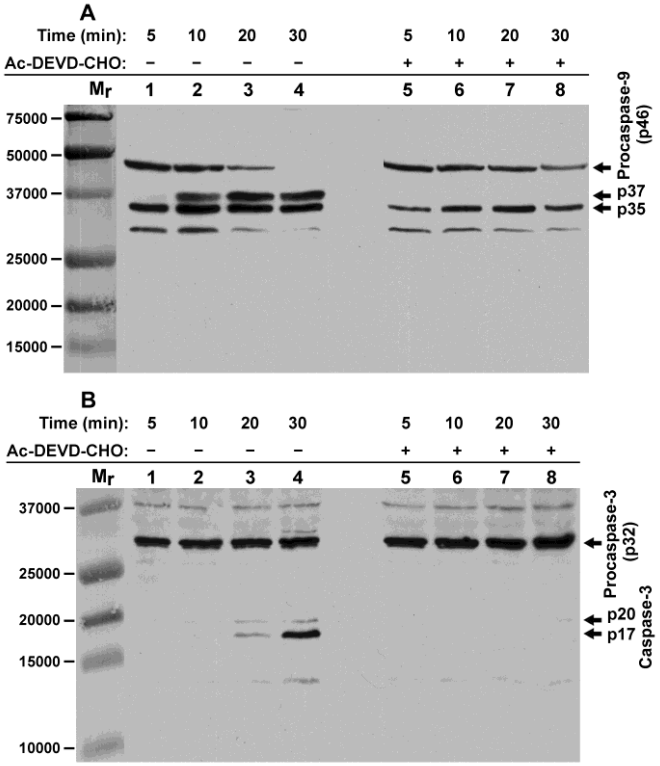

Figure 10. Analysis of procaspase-9 proteolytic processing and caspase-9 activity in NCI-H520 cytosol.

NCI-H520 cell-free cytosol was incubated with cyt-c and dATP and without or with the caspase inhibitor Ac-DEVD-CHO for indicated times. Samples were subjected to SDS-PAGE and were immunoblotted with specific antibodies. (A) The arrows point to procaspase-9 (p46) and the caspase-9 p35 and p37 forms. (B) The arrows indicate procaspase-3 (p32) and the active caspase-3 p20 and p17 forms.

different temperatures and for different times, following the standard incubation with exogenously added cyt-c and dATP at 37˚C for 30 min. As shown in Figure 11, the preincubation at 37˚C for 10 min lead to irreversible loss of the AA activatability in all cell-

free cytosol samples tested, including cytosols from six cell lines, four NSCLC tumours of different histopathological types as well as lung parenchyma (Figure 11). These results provide evidence that the loss of AA activatability by thermal treatment of the *ex-vivo* cell-free cytosol is a general phenomenon and must be considered as a critical drawback condition for biochemical experiments addressing mechanisms of AA regulation.

Apoptosome apparatus in cytosol from NSCLC tumours and its relationship to XIAP

In order to analyse the capability of AA to be activated in NSCLC tumours, we studied first the activatability of AA by cyt-c and dATP in cytosol samples prepared from NSCLC and lung tissues using the fluorometric Ac-DEVD-AFC cleavage assay, which measures the AA-generated caspase-3-like activity. The endogenous (E) as well as the total (T; T = E + I) and the (cyt-c + dATP)-induced incremental (I; I = T - E) caspase-3-like activities were significantly higher in NSCLC tumours as compared to the lungs (Table III). When only the

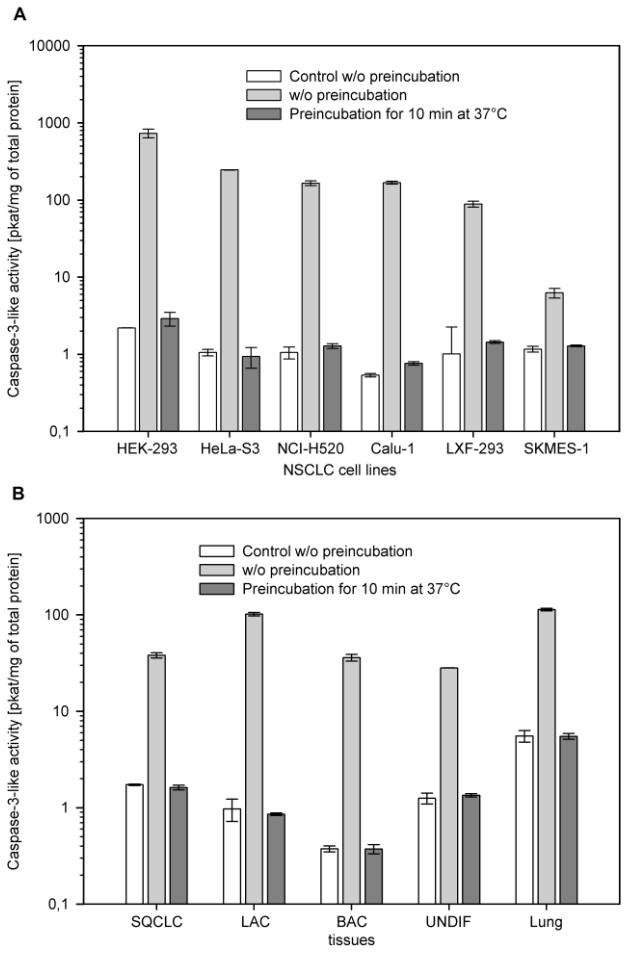

Figure 11. Effect of thermal pretreatment of cell-free cytosols from cell lines, NSCLC tumours and lung parenchyma on the (cyt-c + dATP)-induced caspase-3-like activity.

Cell-free cytosols were incubated without (white) or with (light grey) cyt-c and dATP or were preincubated without cyt-c and dATP at 37˚C for 10 min following addition of cyt-c and dATP and incubation at 37˚C for 30 min (dark grey). Enzymatically released AFC was measured fluorometrically as described in Matherials and Methods. Data represents means ± standard error of the mean from three independent experiments.

robust activation (R) of the (cyt-c+dATP)-induced caspase-3-like activity over the endogenous caspase-3-like activity (*i.e.* $R \geq 2$) was considered, it was found in 19 (31%) of 62 examined tumours and in 5 (8%) of 62 examined lungs ($P = 0.0031$; Chi-square test). When we considered the robust and the weak degrees of AA activation together (*i.e.* $I/E > 0$), then the AA activation was found in 47 (76%) tumours and 29 (47%) lungs ($P = 0.0017$; Chi-square test). These data indicate that AA is more frequently activated in the cytosol from NSCLC tumours compared to the cytosol from lungs.

We further tested whether XIAP played a role in inhibition of AA activity in the cytosol from NSCLC tumours which showed activation response to exogenous cyt-c and dATP. Although there was no inverse correlation between the XIAP protein level and the (cyt-c + dATP)-induced caspase-3-like activity in cytosols from NSCLC tumours.

To test whether the failure to activate the AA in cytosol from NSCLC tumours involves inhibition by XIAP, we used XIAP neutralizing peptides AVPIAQK (P1, a Smac-mimetic peptide) and ATPFQEG (P2, a caspase-9 p12-mimetic peptide), which displayed similar binding affinities toward their target XIAP-BIR3 recombinant polypeptide (Denault *et al*, 2007). Using cytosol samples from NSCLC tumours (n = 21), in which AA was activatable

by cyt-c and dATP, the endogenous caspase-3-like activity (E) was slightly but significantly increased in the presence of peptide P1, whereas its increase by the peptide P2 was not significant ($P = 7.0 \times 10^{-4}$ and $P = 0.082$, respectively; Wilcoxon matched pair test). On the other hand, the (cyt-c + dATP)-induced caspase-3-like activity (I) was significantly increased by the peptide P2 (I-P2), while its increase by the peptide P1 (I-P1) was not significant ($P = 0.012$ and $P = 0.418$, respectively; Wilcoxon matched pair test). In the cytosol from NSCLC tumours, the caspase-3-like activity ratios I-P1/I and I-P2/I higher than 1.5 were found in 4 (21%) and 9 (47%) of 19 examined NSCLC tumours, respectively (Figure 12). Although the (cyt-c + dATP)-induced caspase-3-like activity was more frequently increased by the peptide P2 compared to the peptide P1, this difference was not statistically significant ($P = 0.171$, Chi-square test).

Table III. Analysis of the (cyt-c + dATP)-induced activation of apoptosome apparatus in cytosol from non-small cell lung carcinoma and lung tissues via measurement of increase of the endogenous caspase-3-like activity.

Caspase-3-like activity[a,b] Category of caspase-3-like activity	**Statistical (pkat/mg of total protein)**		**difference (*P*) of caspase-3-like activity in Tumours *vs*. Lungs[c]**
	Tumours	**Lungs**	
Endogenous (E)	**0.717 (0-7.710)**	**0.019 (0.1.351)**	**9.2×10^{-11}**
Total (T)	**1.428 (0-88.706)**	**0.064 (0-5.645)**	**9.8×10^{-11}**
Incremental induced (I)[d]	**0.289 (0-88.429)**	**0.004 (0-5.245)**	**1.3×10^{-5}**
Activity ratio I/E[e]	**1.068 (0.032-536.343)**	**0.488 (0.001-28.396)**	

[a] A total of 62 NSCLC patients was studied including 29 patients with SQCLC, 26 patients with LAC, 1 patient with SQCLC+LAC mixed type tumour, 1 patient with LCLC, 3 patients with SLC, and 2 patients with UNDIF.

[b] Data are represented as the median with the range in parentheses.

[c] Statistical difference of caspase-3-like activity in Tumour *versus* Lungs was calculated using Mann-Whitney test.

[d] Incremental induced activity was calculated as follows: total activity - endogenous activity.

[e] The positive ratio I/E could be calculated in 47 tumours and 29 lungs. Chi-square test showed that the apoptosome apparatus was more frequently activated in tumour cytosols than in lung cytosols ($P = 0.0017$).

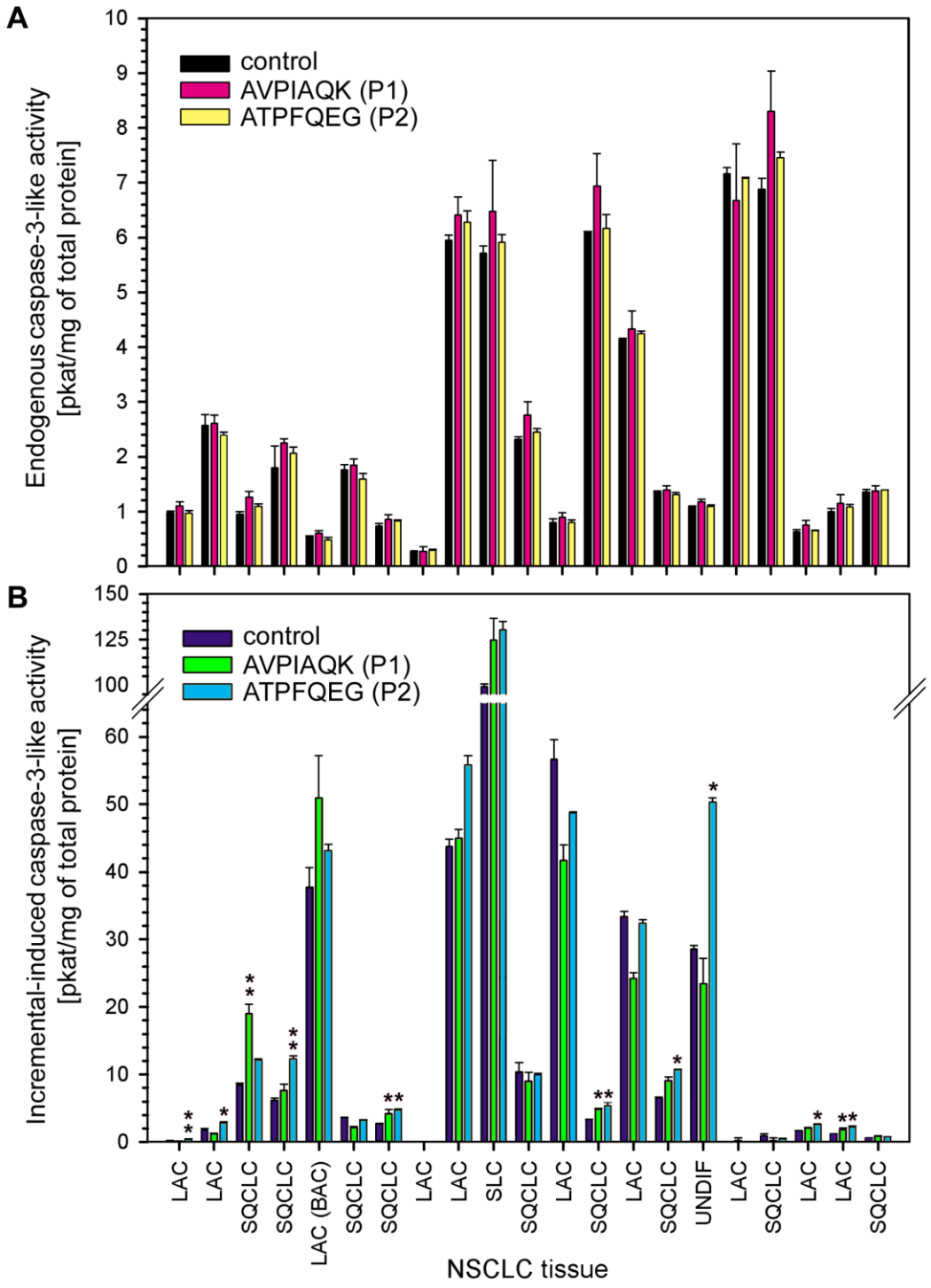

Figure 12. Effect of XIAP-neutralizing peptides on the endogenous and the (cyt-c + dATP)-induced caspase-3-like activity in cell-free cytosols from NSCLC tumours.
Cell-free cytosol samples from 21 selected NSCLC tumours were incubated at 37°C for 30 min (A) alone (black), with P1 (purple), with P2 (yellow), and (B) with cyt-c+dATP (violet), with cyt-c+dATP+P1 (green) and with cyt-c+dATP+P2 (blue). The incremental-induced caspase-3-like activities were calculated as described in Materials and Methods. Data indicate mean ± SEM from three independent experiments. The caspase-3-like activity ratios (I-P1/I and I-P2/I) higher than 2 (**), and >1.5 but <2 (*) are indicated.

5 DISCUSSION

One of the most interesting proteins that regulate the apoptosome pathway is survivin, a member of the IAPs family. Since its discovery by Ambrosini and colleagues in 1997, it was found that this 17 kDa protein is overexpressed in most cancer types. Consistent with previously reported and recent studies (Falleni *et al*, 2003; Nakano *et al*, 2005; Rosato *et al*, 2013), we found that both survivin mRNA and protein are overexpressed in NSCLC cell lines and NSCLC tissues of different histopathological subtypes, while survivin mRNA was significantly higher in SQCLCs as compared to LACs.

It was shown, that survivin only in complex with HBXIP binds procaspase-9 and prevents its recruitment to apoptosome (Marusawa *et al*, 2003). We found that HBXIP mRNA is abundantly expressed in NSCLC cells and tumours as well as in the lungs. This result together with the differential expression of survivin in lung tumours and lungs indicates that survivin is the limiting factor for the formation of the antiapoptotic survivin•HBXIP complex, which may be preferentially generated in lung tumours to inhibit the apoptosome pathway of apoptosis.

XIAP, a member of the IAPs family, which can inhibit the apoptosome pathway on both the initiation and the execution levels (Scott *et al*, 2005; Twiddy *et al*, 2006; Denault *et al*, 2007), showed a slightly but significantly increased expression in NSCLC tumours compared to the lungs in a group of 34 NSCLC patients (Hofmann *et al*, 2002). In a larger subsequent study, we found that the expression of XIAP mRNA in NSCLC cell lines (n = 10) and matched pairs (n = 150) of NSCLC tumours and lungs was not significantly different. However, we demonstrated a significantly increased level of XIAP protein in LAC tumours, as compared to the lungs. The latter results and the lack of correlation between the levels of XIAP mRNA and protein in the tumours suggest that the expression of XIAP may be translationally upregulated and/or post-translationally stabilised in the tumours. In fact, the translation initiation of XIAP mRNA is internal ribosome entry segment (IRES)-dependent and can be enhanced by several IRES *trans*-acting factors such as La autoantigen, hnRNP C1/C2 and MDM2 protein (Holcik *et al*, 2000 and 2003; Gu *et al*, 2009). Furthermore, the level of XIAP in cancer cells can be upregulated through protection against the proteasomal degradation-promoting ubiquitination. Such stabilisation involves phosphorylation of XIAP by the Akt kinase (Dan *et al*, 2004) or interaction of XIAP with survivin (Dohi *et al*, 2004), Notch receptor (Liu *et al*, 2007) or $p34^{SEI-1}$ protein (Hong *et al*, 2009). The upregulated expression of XIAP in cancer cells that occurs in response to DNA damage is due to the Che-

1 protein- (Bruno *et al*, 2008) and/or MDM2 protein-mediated (Gu *et al*, 2009) activation of XIAP NF-κB-dependent transcription and XIAP IRES-dependent translation, respectively.

Although the expression of XIAP and cIAPs in NSCLC tumours does not predict response to classical chemotherapy in patients with advanced NSCLC (Ferreira *et al*, 2001b; Chen P. *et al*, 2010; Seeger *et al*, 2010), treatment of NSCLC cells as well as other cancer cells with novel synthetic Smac-mimetic antagonists of XIAP and cIAPs induces apoptosis in these cells (Lu *et al*, 2008; Galban *et al*, 2009; Petersen *et al*, 2008; Sun *et al*, 2008; Darding *et al*, 2011) or sensitises them against apoptosis inducers such as nonsteroidal anti-inflammatory drugs and TRAIL (Bank *et al*, 2008; Vogler *et al*, 2009). We found, that a peptide inhibitor of XIAP (ATPFQEG, a caspase-9 p12-mimetic peptide) slightly but significantly increased the (cyt-c+dATP)-induced caspase-3-like activity in NSCLC tumpour cytosols. Furthermore, in NSCLC tumour cytosols, XIAP protein levels positively correlated with the endogenous caspase-3-like activity, which itself was slightly but significantly increased in the presence of a Smac-mimetic peptide AVPIAQK.

Surprisingly, NSCLC tumours of different histopathological type showed significantly lower expression of both APIP and UACA mRNAs and proteins as compared to matched lungs. In particular, the expression of UACA mRNA was down-regulated with a high frequency in NSCLC tumours. We frequently observed decreased expression of APIP mRNA and protein in NSCLC tumours. The down-regulation of *APIP* gene expression in NSCLC cells and tissues suggests that APIP is probably not involved in suppression of the apoptosome apparatus in these cancer cells. However, APIP may have antiapoptotic effect in other tumour cells such as head and neck squamous cell carcinoma of tongue and larynx where its expression is up-regulated and associates with increased copy number of *APIP* gene (Järvinen *et al*, 2008).

Immunohistochemical studies of stage I NSCLC tumours demonstrated cytoplasmic localization of Apaf-1 (Apaf-1^{Cyt}) in most examined tumours and marked nuclear localization of Apaf-1 (Apaf-1^{Nuc}) in a small subset (24%) of the tumours (Besse *et al*, 2004). Interestingly, the Apaf-1^{Nuc} group of NSCLC patients had significantly better overall survival rate as compared to the Apaf-1^{Cyt} group (Besse *et al*, 2004). Recently, a non-apoptotic role for the intranuclear Apaf-1 in the DNA damage checkpoint activation has been demonstrated (Zermati *et al*, 2007). As increased expression of UACA/nucling has been implicated in promoting Apaf-1 nuclear translocation after proapoptotic stress (Sakai *et al*, 2004), the nuclear translocation of Apaf-1 may be impaired in the majority of NSCLC tumours due to down-regulation of UACA expression.

UACA/nucling has been shown to interact with the transcription factor NF-κB and to prevent its nuclear translocation thus reducing expression of the NF-κB-targeted genes (Liu *et al*, 2009). Under the conditions of down-regulated expression of UACA in NSCLC cells, we may expect increased nuclear translocation of NF-κB and up-regulation of NF-κB-targeted genes, which were shown to mediate cancer cells survival (Chaturvedi *et al*, 2011; Chen *et al*, 2011b).

We found significant upregulation of Apaf-1XL/LC activatable variants, as well as procaspase-9 variant in NSCLC tumours and lungs. These results, together with the previous reports demonstrating that NSCLC cells and tumours express increased or sufficient levels of Apaf-1, procaspase-9 and procaspase-3 mRNAs and proteins compared to lungs (Krepela *et al*, 2004 and 2006), predicts that NSCLC tissues are advantageously equipped with the key apoptosome components for inducing apoptosis. However, the induction of functional AA in terms of its capability to generate robust caspase-3-like activity was missing in 3 of 7 investigated NSCLC cell lines and 43 (69%) of 62 examined NSCLC tumours. This suggests that certain NSCLC cell populations possess mechanisms that avoid or suppress the functional AA. Analysis of the (cyt-c+dATP)-activated cell-free cytosol from A549 cells failed to detect the caspase-9 p35 the p37 subunits in the high-M_r fractions where Apaf-1 protein and procaspase-9 coeluted, thus forming a non-functional AA complex of ~1400 kDa. Similar results were reported in A549 cells by Hoffarth *et al* (2008). However, we could detect the p35 and p37 caspase-9 subunits in A549 cell-free cytosol regardless of the addition of cyt-c and dATP, when much higher concentration of the anti-caspase-9 antibody was used. Although the origin of the caspase-9 p35 and p37 forms in A549 cells cytosol is not known, for instance, calpains are capable of procaspase-9 cleavage to p35 and p37 caspase-9 forms (Chua *et al*, 2000). In the (cyt-c+dATP)-activated A549 cells cytosol, Apaf-1 monomer was able to oligomerize and bind procaspase-9, but the processing of the apoptosome-associated procaspase-9 was blocked. Moreover, most of the procaspase-9 was present outside the apoptosome. The trace amounts of p35 and p37 caspase-9 forms detected in the apoptosome did not have to be generated by the apoptosome itself, but as extra- apoptosome generated caspase-9 forms they could merely be recruited to the apoptosome via their CARD domains. It was already demonstrated that the p35 and p37 caspase-9 forms could be recruited to apoptosome (Zou *et al*, 2003).

The reason(s) for the blocked processing of the apoptosome-associated procaspase-9 in A549 cells are not known yet. The possible candidate mechanisms for suppression of the

apoptosome-associated procaspase-9 activation might involve the inhibitory phosphorylations of procaspase-9 (Kurokawa and Kornbluth, 2009; Allan and Clarke, 2009). However, this seems also to be unlikely, because APIP protein, the activator of Akt and Erk1/ 2 protein kinases that can introduce the inhibitory phosphorylations into procaspase-9 (Cho *et al*, 2007), is strongly downregulated in NSCLC cells and tumours (Moravcikova *et al*, 2012).

Importantly, in the (cyt-c+dATP)-activatable cell-free cytosols from NSCLC cell lines two distinct mechanisms can be seen in procaspase-9 processing. The first one involves procaspase-9 monomer autoprocessing to a caspase-9 form having the p35 and p12 subunits. The second one is a feedback processing mediated by caspase-3 that cleaves procaspase-9 monomer to a caspase-9 form containing the p37 and p10 subunits. Generation of the p35 caspase-9 form precedes the formation of the p37 caspase-9 form, which starts to be produced once the apoptosome-associated caspase-9 p35/p12 form proteolytically converts procaspase-3 to active caspase-3 (Zou *et al*, 2003; Denault *et al*, 2007).

Interestingly, both, the processing of procaspase-9 to its p37 form and the generation of active caspase-3 were completely blocked by the caspase aldehyde inhibitor Ac-DEVD-CHO. Thus, the inhibitor does not inhibit the apoptosome-dependent procaspase-9 activation to caspase-9 p35 form, but it inhibits the apoptosome-associated caspase-9 p35 form. This finding is consistent with a study by Srinivasula and colleagues (Srinivasula *et al*, 1998).

Furthermore, we provided evidence that mere incubation of cell-free cytosol from COLO-699 cells without cyt-c and dATP leads to formation of an aggregate of Apaf-1 monomers, which is not able to recruit and activate procaspase-9. However, formation of Apaf-1 aggregates is an irreversible process, which extent increases with time and temperature of incubation in all studied cell and tissue cytosols including cytosols prepared from NSCLC tumours and lungs. The irreversible inactivation of Apaf-1 in the absence of dATP was also observed by Kim *et al*, 2005. Therefore, preincubation of cell-free cytosol intended to reactivate modified AA component without simultaneous addition of cyt-c+dATP certainly leads to irreversible loss of AA activatability, unless the temperature is kept at 4 ^{o}C or below (Cardone *et al*, 1998; Kim *et al*, 2008). The aggregation and hence inactivation of Apaf-1 at 37°C does not occur *in vivo*. Although the reason for that event is still not clear yet, it probably involves sustained (d)ATP sufficiency in the living cell. It seems that (d)ATP might play an important role in maintaining Apaf-1 in a cyt-c-activatable state (Zhang *et al*, 2011). By contrast, procaspase-2 activation platform, called PIDDosome, can assemble from monomeric components, such as PIDD-1 and RAIDD/CRADD, into a functional complex spontaneously at 37°C (Tinel and Tschopp, 2004).

6 CONCLUSION

The results of this study suggest a higher predisposition to AA-mediated apoptosis in NSCLC tumours than in lungs. Similar findings were reported for breast carcinomas and brain tumours compared to their counterpart normal tissues (Schafer *et al*, 2006; Johnson *et al*, 2007). The higher propensity of NSCLC tumours to apoptosome-mediated apoptosis is due to several factors. First, because of increased expression of activatable apoptosome pathway core protein components in NSCLC tumours, including Apaf-1 and procaspases-9 and -3. Second, because of the down-regulation of expression of *APIP* and *UACA* genes causing the lack of APIP-mediated AA suppression and UACA-assisted Apaf-1 nuclear entry, which would lead to the failure of DNA damage checkpoint activation in NSCLC cells leading to their genomic instability and contributing to development and progression of NSCLC tumours. However, the functionality of AA is suppressed in some NSCLC cell lines and in a high proportion of NSCLC tumours. In conclusion, our results indicate that the XIAP-mediated inhibition is not the major suppressor mechanism of apoptosome pathway induction in NSCLC tumours and that failure of the apoptosome-bound procaspase-9 activation may underlie the malfunction of apoptosome pathway in NSCLC cells.

7 LIST OF PUBLICATIONS

PAPERS RELATED TO THE THESIS

E. Moravcikova, E. Krepela, J. Prochazka, Cermak, and K. Benkova. Apoptosome apparatus in non-small cell lung carcinoma. Submitted to *British J. Cancer*.

E. Moravcikova, E. Krepela, J. Prochazka, I. Rousalova, J. Cermak, and K. Benkova. 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 = 2.399)

E. Krepela, P. Dankova, **E. Moravcikova**, A. Krepelova, J. Prochazka, J. Cermak, J. Schutzner, P. Zatloukal, and K. Benkova. Increased expression of inhibitor of apoptosis proteins, survivin and XIAP, in non-small cell lung carcinoma. *Int.J.Oncol.* 35 (6):1449-1462, 2009. (IF = 2.399)

PUBLISHED PAPERS

L. Janovec, D. Sabolova, M. Kozurkova, H. Paulikova, P. Kristian, J. Ungvarsky, **E. Moravcikova**, M. Bajdichova, D. Podhradsky, J. Imrich. Synthesis, DNA interaction, and cytotoxic activity of a novel proflavine-dithiazolidinone pharmacophore. *Bioconjug.Chem*. 18(1):93-100, 2007. (IF = 4.930)

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E. Moravcikova, E. Krepela, J. Prochazka, J. Cermak, and K. Benkova. Expression and activation of the appoptosome apparatus core components in non-small cell lung carcinoma. 22nd IUBMB and 37th FEBS Congress, Sevilla, Spain. *FEBS J*. 279(s1):52, 2012.

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