Increased expression of inhibitor of apoptosis proteins, survivin and XIAP, in non-small cell lung carcinoma

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Received June 5, 2009; Accepted August 7, 2009

DOI: 10.3892/ijo_00000464

Abstract. Members of the inhibitor of apoptosis protein (IAP) family, survivin and X-chromosome-linked IAP (XIAP), contribute to apoptosis resistance of cancer cells, and an increase in their expression may elevate the apoptotic threshold of malignant tumours during their growth and progression. In the present study, we investigated the expression status of survivin and its interactants hepatitis B X-interacting protein (HBXIP) and XIAP in non-small cell lung carcinoma (NSCLC) cell lines and NSCLC tumours and matched lungs from surgically treated patients in relation to their clinicopathological data. The expression of survivin, HBXIP and XIAP mRNAs was quantitated by real-time RT-PCR. The expression of survivin and XIAP proteins was analysed by Western blotting and ELISA. Survivin mRNA and protein levels were highly upregulated in NSCLC cells and tissues as compared to the lungs. In fact, the levels of survivin mRNA and protein in the tumours were more than 10-fold higher in 96 (64%) and 72 (82%) of the 150 and 88 examined NSCLC patients, respectively. The expression of survivin mRNA was higher in squamous cell lung carcinomas than in lung adenocarcinomas (LACs; P=0.003) and in less-differentiated tumours than in well-differentiated ones (P=0.007). The level of survivin protein was higher in stage IB and stage II+III tumours (P=0.049 and P=0.044), than in stage IA tumours. The *BIRC5* promoter polymorphism at nucleotide -31 did not influence the expression of survivin mRNA and protein in NSCLC cells and tumours. HBXIP mRNA was abundantly expressed in NSCLC cell lines and NSCLC tumours and lungs, while its level was comparable in the tumours and lungs. The expression of XIAP mRNA in NSCLC cell lines and NSCLC tumours and lungs was not significantly different. However, the expression of XIAP protein was higher in NSCLC tumours, particularly in LACs, as compared to the lungs (P=0.017 and P=0.004). In conclusion, the overexpression of survivin in the majority of NSCLCs together with the abundant or upregulated expression of HBXIP and XIAP suggest that tumours are endowed with resistance against a variety of apoptosis-inducing conditions.

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Key words: non-small cell lung carcinoma, survivin, hepatitis B X-interacting protein, X-chromosome-linked inhibitor of apoptosis protein, expression, apoptosis

Introduction

The human inhibitor of apoptosis protein (IAP) family encompasses eight members: NAIP (BIRC1), cIAP1 (BIRC2), cIAP2 (BIRC3), XIAP (BIRC4), survivin (BIRC5), apollon (BIRC6), livin (BIRC7) and ILP-2 (BIRC8) (1,2). These proteins participate in the regulation of a variety of cell functions including apoptosis, cell division, non-apoptotic signal transduction and copper homeostasis (1,2). IAPs block apoptotic cell death primarily via mechanisms that repress the activation and activity of certain caspases and/or reduce their protein levels. These mechanisms involve prevention of the activation of procaspase-9 (3) and -8 (2,4), inhibition of the activity of caspase-9, -3 and -7 (5-9), and ubiquitination of active caspase-9, -3 and -7 to promote their proteasomal degradation (10-14). Moreover, various IAPs such as XIAP, cIAP1, cIAP2, apollon and livin can bind to the endogenous IAP-antagonists Smac (15) and Htra2 (16) and ubiquitinate them to facilitate their degradation by proteasomes (10,14,17-20). Besides their caspase-, Smac- and Htra2-directed antiapoptotic activities, both cIAPs and XIAP are capable of activating the nuclear factor (NF)-κB signaling pathways and thereby generate a series of prosurvival responses in cancer cells as well as in vascular endothelial cells (1,2,4,21-23).

Resistance to apoptosis contributes to cancerogenic transformation of cells and tumourigenesis as well as to malignant progression of tumours and lack of their responsiveness to non-surgical modes of therapy (24-26). Although the overexpression of multiple IAPs has been implicated in apoptosis

resistance of human neoplasms (2,27-30), recent data indicate that high expression of both survivin and XIAP is particularly critical for apoptosis suppression in cancer cells derived from solid human tumours (31-36). Moreover, survivin and XIAP are also upregulated in angiogenic factor-activated endothelial cells of tumour microvasculature and protect them from endogenous and exogenous apoptotic stimuli (37-40).

Survivin and XIAP have been implicated as resistance factors in the drug- and radiation-induced apoptosis of non-small cell lung carcinoma (NSCLC) cells (41-45). Based on these data, there is a possibility that both survivin and XIAP may contribute to tumourigenesis and progression of NSCLCs due to their apoptosis suppression functions. Therefore, in the present study, we investigated the expression status of survivin and its antiapoptotic-function-promoting interactants hepatitis B X-interacting protein (HBXIP) and X-chromosome-linked IAP (XIAP) in NSCLC cell lines and different subtypes of NSCLC tumours and lungs from surgically treated patients in relation to clinicopathological data.

Materials and methods

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

Patients and tissues. The patients (n=150) (median age, 62 years; range, 39-78 years; 110 men and 40 women; 130 smokers and 20 non-smokers) entering the study had not received radiotherapy or chemotherapy before surgery for lung cancer. The surgical treatment applied to the patients included lung lobectomy, bilobectomy or pneumonectomy, and regional lymph node dissection. The histopathological classification of lung tumours was carried out according to the World Health Organization criteria (46), and the following NSCLC types were included in the present study: squamous cell lung carcinoma (SQCLC, n=69), lung adenocarcinoma (LAC, n=56), SQCLC+LAC mixed-type tumours (n=3), large-cell lung carcinoma (LCLC, n=7), sarcomatoid lung carcinoma (SLC, n=4) and undifferentiated lung carcinoma (UNDIF, n=11). Tumour staging was performed according to the International pTNM Staging System (47). Written informed consent was obtained from each patient before entry into the study. The study was approved by the local institutional ethics committee.

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

Isolation and quantification of DNA. Genomic DNA was isolated from cultured NSCLC cell lines and NSCLC tissue samples using the Puregene DNA Isolation Kit (cat. no. D-7000a; Gentra Systems, Minneapolis, MI, USA) according to the manufacturer's guidelines. The concentration of DNA was determined by spectrophotometry at 260 nm in 10 mM Tris/HCl buffer, pH 7.5. Preparations of DNA in the Hydration Solution (a Tris/EDTA buffer) were stored in small aliquots at -78˚C until analysis.

DNA sequencing. Genotyping of the survivin gene (*BIRC5*) promoter for the polymorphism at nucleotide (NT) -31 in NSCLC cell lines and NSCLC tissues was performed using direct DNA sequencing, in both forward and reverse directions, of purified PCR products after specific PCR amplification of a survivin promoter region from NTs -510 to +40 using genomic DNA as the template. DNA amplification was carried out in a 30-μl PCR reaction containing 10 mM Tris/HCl buffer, pH 8.8, 50 mM KCl, 0.08 v/v% Nonidet NP40, 200 nM of each dGTP, dCTP, dATP and dTTP, 1 mM $MgCl_2$, 15 pmol of the amplification primers 5'-CAATCTC AGCTCACTGCACCCTCT-3' (forward) and 5'-GAAAG GGCTGCCAGGCAGGGGGCAA-3' (reverse) (48), 100 ng of genomic DNA, and 1 unit of Taq DNA Polymerase (Fermentas, Vilnius, Lithuania). The amplification consisted of 32 cycles of denaturation at 95˚C for 1 min, annealing at 58˚C for 1 min, and extension at 72˚C for 2 min, followed by a final extension at 72˚C for 7 min. The PCR products were purified using the SureClean PCR purification kit (Bioline, London, UK) according to the recommendations of the supplier. DNA sequencing was carried out using the purified PCR product, BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and the nested sequencing primers 5'-AGCTCACTGCACCCTCTGCC-3' or 5'-CTGCCAGG CAGGGGGCAACG-3'. Sequence analysis was performed on the 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

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

Transcript	GeneBank accession no.	Sequences of primers and TaqMan probe		Final concentration
Survivin	NM_001168	Forward primer:	5'-GACGACCCCATAGAGGAACATA-3'	400 nM
		Reverse primer:	5'-TTTCCTTTGCAATTTTGTTCTTG-3'	400 nM
		TaqMan probe:	5'-(6-FAM) CCGGTTGCGCTTTCCTTTCTGTCA (TAMRA)-3'	200 nM
HBXIP	NM_006402	Forward primer:	5'-AGCACTTGGAAGACACAATGAAG-3'	400 nM
		Reverse primer:	5'-GTTTCTGGATCATAATGTTCCCAT-3'	400 nM
		TaqMan probe:	5'-(6-FAM) CCGCGGGACCCTGTCAGATGA (TAMRA)-3'	200 nM
XIAP	U45880	Forward primer:	5'-TCCAGAATGGTCAGTACAAAGTTG-3'	200 nM
		Reverse primer:	5'-TTTGTTGAATTTGGGAAATTCCT-3'	200 nM
		TaqMan probe:	5'-(6-FAM) CACTTCGAATATTAAGATTCCGGCCCA (TAMRA)-3'	200 nM
ß-actin	NM_001101	Forward primer:	5'-CTGGCACCCAGCACAATG-3'	200 nM
		Reverse primer:	5'-GGGCCGGACTCGTCATAC-3'	200 nM
		TaqMan probe:	5'-(VIC) AGCCGCCGATCCACACGGAGT (TAMRA)-3'	200 nM

Isolation and quantification of total RNA. Total RNA was isolated from tumour and lung tissue samples and from cultured NSCLC and SCLC cell lines using the Trizol Reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Spectrophotometric analysis, which was carried out in 10 mM Tris/HCl buffer, pH 7.5, revealed that all samples of total RNA had an A_{260nm}/A_{280nm} ratio >1.8. The concentration of total RNA was determined by fluorometry using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. The preparations of total RNA in RNase-free water (ICN Biomedicals, Irvine, CA, USA) were stored in small aliquots at -78˚C until analysis.

Real-time RT-PCR analysis. The sequences and final concentrations of the oligonucleotide primers and probes used in real-time RT-PCR assays of expression of the investigated transcripts are indicated in Table I. The concentrations of primers and TaqMan probes adopted for real-time RT-PCR quantitation of each studied transcript were determined in optimization experiments. The primers and probes were designed with the program Primer Express (Applied Biosystems) and were synthesized at Proligo (Paris, France) and Applied Biosystems (Warrington, Cheshire, UK), respectively.

The expression of survivin mRNA and HBXIP mRNA was quantitated by a two-step real-time RT-PCR assay as follows. In the first step, 2 μg of total RNA was reverse transcribed in a total volume of 20 μl of 50 mM Tris/acetate buffer, pH 8.4, containing 75 mM of potassium acetate, 8 mM of magnesium acetate, 500 nM of the gene-specific reverse primer (Table I), 200 nM of each dGTP, dCTP, dATP and dTTP, 5 mM dithiothreitol, 40 units of RNase inhibitor RNAseOUT (Invitrogen) and 15 units of ThermoScript Reverse Transcriptase (Invitrogen). The RT reaction was carried out at 58˚C for 30 min and was terminated by heating at 85˚C for 10 min. The resulting RT mixes were stored at -25˚C until PCR analysis. The PCR step was carried out in a total volume of 50 μl of 20 mM Tris/HCl buffer, pH 8.4, containing 50 mM of KCl, 1.85 mM of $MgCl_2$, 220 nM of each dGTP, dCTP, dATP and dTTP, the indicated concentrations of gene-specific forward and reverse primers and TaqMan probe (Table I), 2 units of Platinum Taq DNA polymerase (Invitrogen), and 2 μl of the RT mix (representing an equivalent of an input of 200 ng of total RNA). The PCR amplification included a hot start at 95˚C for 3 min and 45 cycles of denaturation at 95˚C for 15 sec and of annealing/extension at 58˚C for 1 min.

The expression of XIAP mRNA was quantitated by a coupled real-time RT-PCR assay as follows. The RT-PCR reaction mixtures had a total volume of 50 μl and contained 25 μl of ThermoScript Reaction Mix (a buffer with 3 mM $MgSO_4$ and 200 nM of each dGTP, dCTP, dATP and dTTP) and 1 μl of ThermoScript Plus Reverse Transcriptase/Platinum Taq DNA Polymerase Mix (both Mixes were from Platinum Quantitative RT-PCR ThermoScript One-Step System, Invitrogen), the indicated concentrations of gene-specific forward and reverse primers and TaqMan probe (Table I), 40 units of RNaseOUT (Invitrogen), and an input of 200 ng of total RNA.

The expression of ß-actin mRNA (an internal reference transcript) was quantitated in parallel to the indicated target transcripts either by the two-step or by the coupled real-time RT-PCR assay as described above. The respective gene-specific forward and reverse primers and TaqMan probe and their concentrations in the PCR assays were as indicated in Table I.

The real-time RT-PCR assays were run in triplicate or duplicate in MicroAmp Optical 96-well Reaction Plates on the ABI PRISM 7700 Sequence Detection System (SeDeS) operated from within the SeDeS software (all from Applied Biosystems). The threshold cycle (C_T) values of the amplification reactions, represented by the plots of background-subtracted fluorescence intensity (ΔFI) of the reporter dye (6-FAM or VIC) against PCR cycle number, were determined with the SeDeS software. The tumour/lung ratio of the ß-actin mRNA-normalised target transcript expression was calculated by means of the $2^{-\Delta\Delta C_T}$ method (49), and the

statistical difference of the ß-actin mRNA-normalised target transcript expression in tumours and lungs was calculated from the linearized ΔC_T data (i.e. $2^{-\Delta C_T}$) (50).

Preparation of protein extracts and total protein determination. NSCLC cell lines harvested by scraping into the Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution were pelleted by centrifugation at 240 x g and 4˚C for 10 min. The pellets were disrupted in lysis buffers by sonication. For the analysis of survivin protein expression, the lysis buffer was 0.5% Triton X-100, 0.005% Tween-20 and 6 M urea in phosphate-buffered saline, pH 7.2-7.4, containing a mixture of proteinase inhibitors including 1 mM Na_2EDTA, 50 μM E-64, 200 μM AEBSF and 25 μM pepstatin. For the analysis of XIAP protein expression, the lysis buffer was the same except urea. Frozen tissue samples were homogenized on ice in 800 μl of the mentioned lysis buffers using the Ultra-Turrax T25 homogenizer fitted with the probe S25N-8G (Janke and Kunkel, Staufen, Germany). Ten 5-sec probe bursts at 24,000 rpm were applied. The homogenates were centrifuged at 40,000 x g and 4˚C for 30 min, and the collected supernatants, i.e. extracts, were divided into small aliquots and stored at -78˚C until analysis.

Total protein concentration in the extracts was determined by the bicinchoninic acid assay using bovine serum albumin as a standard (51).

Immunoblotting analysis. The expression of survivin and XIAP proteins in NSCLC cells was investigated using denaturing SDS-polyacrylamide gel electrophoresis and immunoblotting with specific antibodies. The electrophoretic separation of extracted proteins (100 μg of per lane) was carried out in 16.5% T/3% C and 12.5% T/3% C polyacrylamide gels, respectively, using the Tris-Tricine-SDS buffer system (52). The Precision Plus Protein Prestained Standards (BioRad, Hercules, CA, USA) were run in parallel to extracted proteins which were preboiled for 5 min in 50 mM Tris/HCl buffer, pH 7.4, containing 2.5% SDS, 100 mM dithiothreitol, 5% glycerol and 0.01% Serva Blue G. The separated proteins were electrotransferred onto sheets of Hybond P membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) using a transfer buffer 48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20 v/v% methanol, pH 9.2. Survivin and XIAP proteins were visualised on the membrane by an immunodetecting procedure coupled to an enhanced chemiluminescence generating system. The specific primary antibodies used were anti-survivin mouse monoclonal IgG_{2a} (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. no. sc-17779) and anti-XIAP rabbit polyclonal IgG (R&D Systems, Minneapolis, MN, USA; cat. no. RD-AF822). The secondary antibodies used were goat anti-mouse IgG_{2a}-horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology) and goat anti-rabbit IgG-HRP peroxidase conjugate (Sigma). The immunoblotting procedure was carried out at room temperature with the following treatment steps: blocking with 5% Blotting Grade Blocker (BGB; BioRad) and 1% bovine serum albumin (BSA) in PBST buffer (20 mM NaH_2PO_4/80 mM Na_2HPO_4, 100 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 h, incubation with the primary antibody (at 1 μg/ml in 1% BGB and 0.2% BSA in PBST buffer) for 2 h, extensive washing with PBST buffer, incubation with the secondary HRP-conjugated antibody (at a dilution of 1:10000 in 1% BGB and 0.2% BSA in PBST buffer) for 1 h, extensive washing with PBST buffer, incubation with the ECL Plus Reagent (Amersham Pharmacia Biotech) for 5 min, and capture of the chemiluminescence signal on BioMax Light-1 film (Eastman Kodak, Rochester, NY, USA). The net intensity of survivin and XIAP protein bands in the film immunograms was evaluated with the 1D Image Analysis Software (Eastman Kodak) as described previously (53).

Determination of survivin and XIAP proteins. The levels of survivin and XIAP proteins in cell and tissue extracts were measured by chromogenic sandwich enzyme-linked immunosorbent assays (ELISAs) using Human Survivin ELISA DuoSet IC (cat. no. DYC647) and Human XIAP ELISA DuoSet IC (cat. no. DYC822) kits (R&D Systems) according to the manufacturer's instructions. For ELISA of survivin protein, 2.5 and 5 μg, 12.5 and 25 μg, and 75 and 150 μg of total extracted protein per microtiter plate well were routinely used in NSCLC cell lines, NSCLC tissues and lungs, respectively. For ELISA of XIAP protein, 5 and 10 μg of total extracted protein per microtiter plate well were routinely used in NSCLC cell lines and tissues as well as in lungs. The intraassay coefficients of variation for ELISAs of low and high concentration of survivin and XIAP in extract samples were 3.1 and 4.9%, and 2.5 and 4.6%, respectively.

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

Results

To analyse the expression status of survivin, HBXIP and XIAP mRNAs in NSCLC cell lines and NSCLC tissues and lungs we quantitated the level of these transcripts and of ß-actin mRNA by means of real-time RT-PCR. A set of PCR amplification plots which is shown in Fig. 1 illustrates overexpression of survivin mRNA in NSCLC tissues as compared to matched lungs and equal or comparable expression of HBXIP and XIAP mRNAs and ß-actin mRNA in tumour-lung matched pairs. Using the two-step real-time RT-PCR assay, there was no significant difference between the expression levels of ß-actin mRNA (2^{-C_T} data) in the investigated NSCLC and SCLC cell lines (P=0.66, Mann-Whitney test) and in NSCLC tissues of different histopathological types and matched lungs (P>0.19; Mann-Whitney test). With the coupled real-time RT-PCR assay, the expression of ß-actin mRNA in NSCLC and SCLC cell lines was not significantly different (P=0.22, Mann-Whitney test), but it was slightly and significantly higher in SQCLC and LAC tissues as compared to matched lungs ($P=4.5x10^{-4}$ and $P=4.1x10^{-4}$, respectively, Mann-Whitney test).

Survivin (*BIRC5*) and XIAP (*BIRC4*) genes were strongly expressed in the studied NSCLC cell lines at both the mRNA and protein levels, and this expression showed a significant

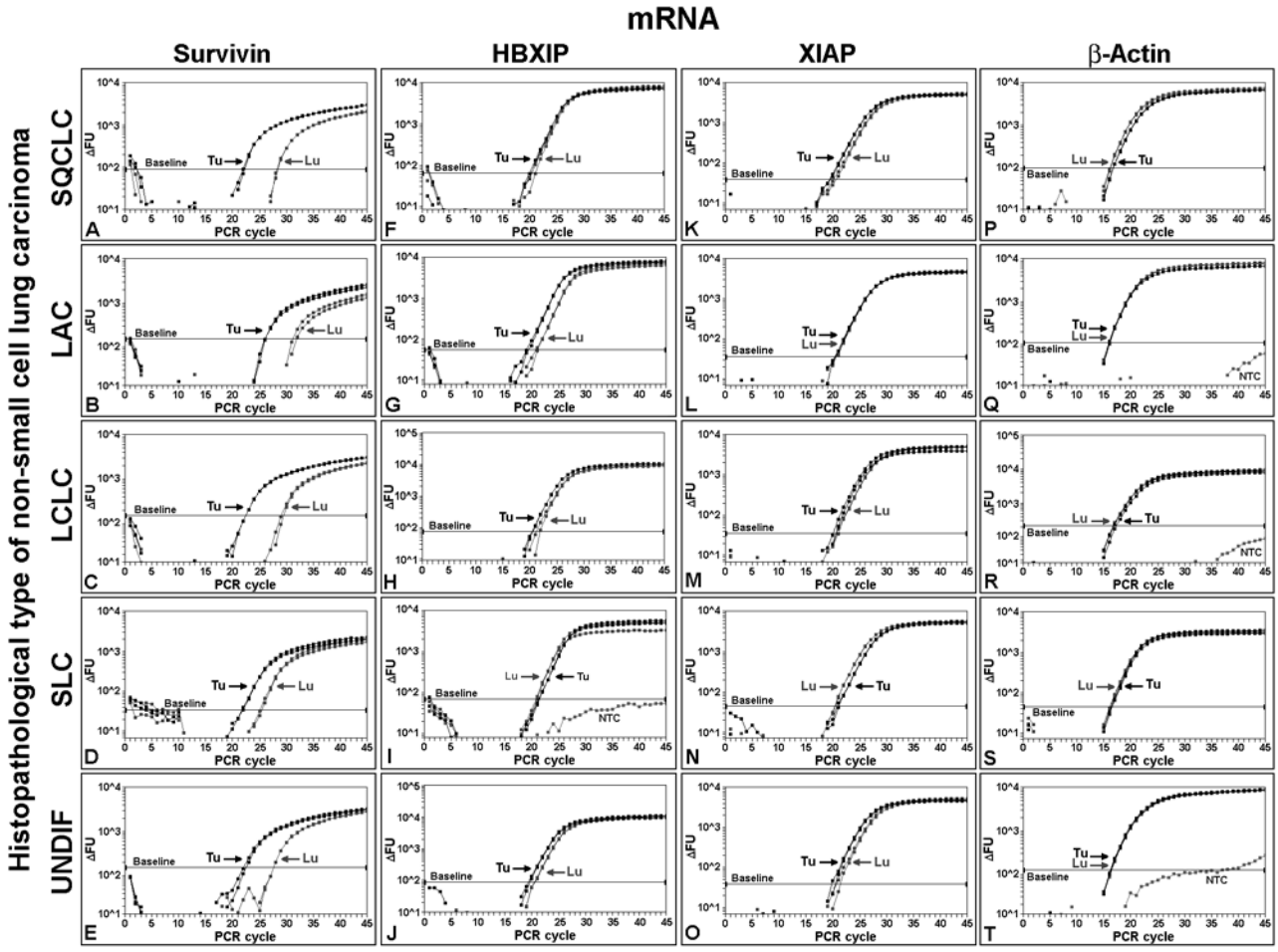

Figure 1. Real-time RT-PCR quantitation of expression of survivin, HBXIP, XIAP and ß-actin mRNAs in non-small cell lung carcinoma tissues and matched lungs. The set of PCR amplification plots, representing the two-step (A-E, F-J and P-T) and the coupled (K-O) real-time RT-PCR assays with an input of 200 ng of total RNA and running in duplicate, shows the expression of survivin, HBXIP, XIAP and ß-actin mRNAs in five tumour (Tu)-lung (Lu) matched pairs. The tumours were: squamous cell lung carcinoma (SQCLC), lung adenocarcinoma (LAC), large-cell lung carcinoma (LCLC), sarcomatoid lung carcinoma (SLC), and undifferentiated lung carcinoma (UNDIF). NTC, no template control. ΔFU, background-subtracted fluorescence intensity (in arbitrary units) of the released reporter dye (6-FAM or VIC).

positive correlation, respectively (Fig. 2). There was no obvious relationship between the genotype at NT -31 in *BIRC5* promoter and the expression of survivin mRNA and protein in the studied NSCLC cell lines (Fig. 2A and B). The level of survivin mRNA, but not of XIAP mRNA, was significantly lower in NSCLC cell lines as compared to SCLC cell lines (Fig. 3). The latter findings confirm data previously reported for the expression of survivin and XIAP mRNAs in panels of different NSCLC and SCLC cell lines (54,55).

The expression of survivin mRNA was substantially higher in NSCLC tissues of different histopathological types as compared to matched lungs (Table II). In fact, in 96 (64%) of the 150 examined NSCLC patients, the tumours had a >10-fold higher level of survivin mRNA. Concerning the histopathological types of NSCLC, the expression of survivin mRNA in LACs was significantly lower as compared to SQCLCs, LCLCs and UNDIFs (Table II). The expression of survivin protein was also highly upregulated in NSCLC tissues as compared to matched lungs (Table III), while the level of survivin protein in the tumours was >10-fold higher in 72 (82%) of the 88 examined NSCLC patients. The expression of survivin mRNA and protein showed a close positive correlation in NSCLC tissues but not in the lungs (Fig. 4). The expression of both survivin mRNA and protein in NSCLC tissues was significantly lower as compared to the studied NSCLC cell lines ($P=7.9x10^{-7}$ and $P=6.4x10^{-5}$, respectively; Mann-Whitney test).

The *BIRC5* promoter polymorphism at NT -31 had no significant impact on the expression of survivin mRNA and protein in NSCLC tissues (Fig. 5). The expression of survivin mRNA, but not of protein, reached a significantly higher level in the tumours of men than women, in the tumours of smokers than non-smokers, and in the less-differentiated tumours (Table IV). On the other hand, the expression of survivin protein, but not of mRNA, was significantly higher in stage IB tumours as well as in higher stage tumours as compared to stage IA tumours (Table IV). When SQCLCs and LACs were considered separately, the expression of survivin mRNA remained significantly higher in the less-differentiated tumours (P=0.035 and P=0.008, respectively; Mann-Whitney test), but it became statistically insignificantly different in the tumours of men and women and the tumours

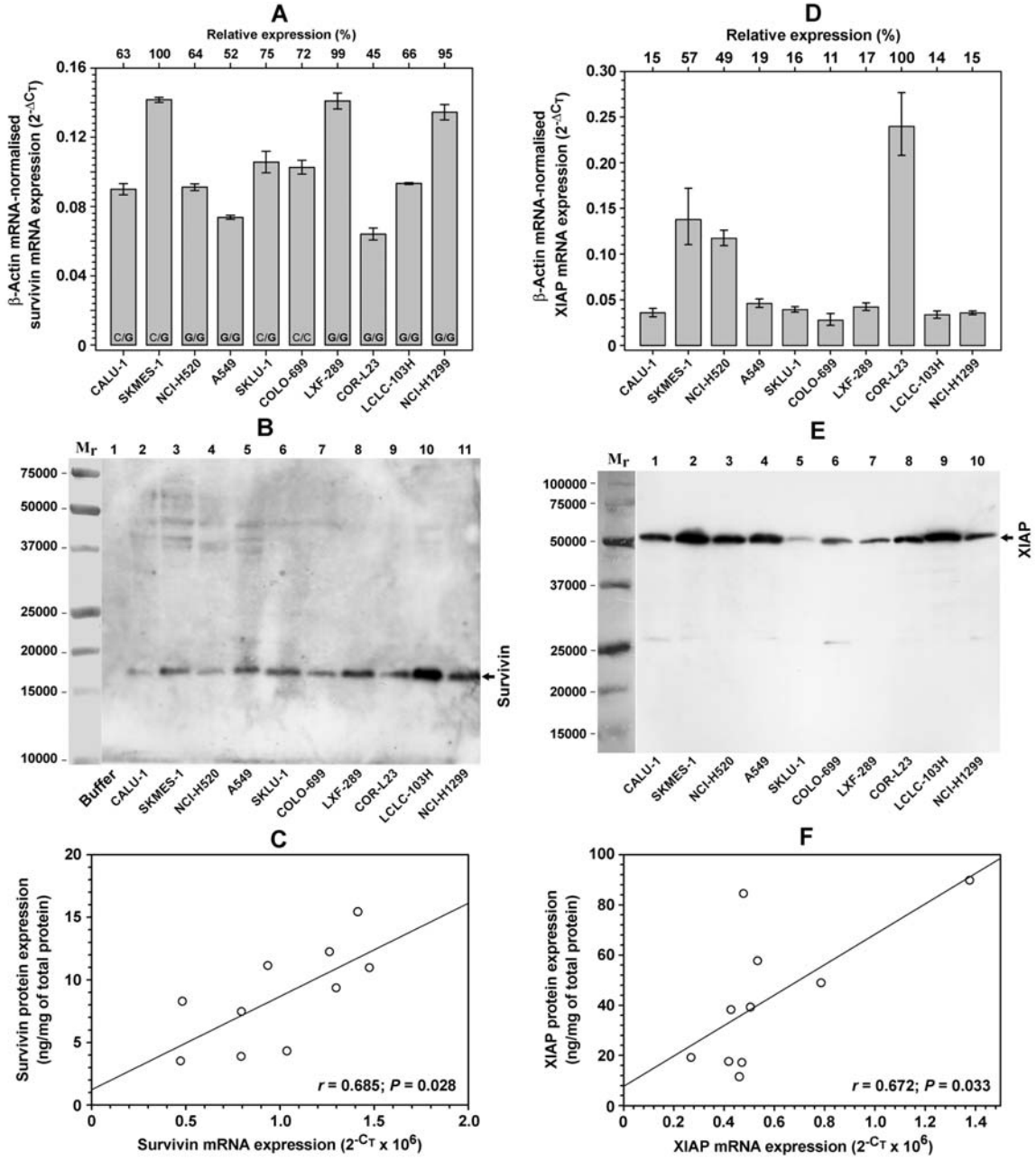

Figure 2. Analysis of survivin and XIAP expression in non-small cell lung carcinoma cell lines. (A and D) Expression of survivin and XIAP mRNAs in the tumour cell lines as quantitated by real-time RT-PCR. Data are represented as the mean ± standard error of the mean of three independent experiments. At the bottom of each column, the genotype at nucleotide -31 in the survivin gene (*BIRC5*) promoter is shown. (B and E) Expression of survivin and XIAP proteins in the tumour cell lines as analysed by SDS-PAGE and immunoblotting. (C and F) Correlation analysis of mRNA and protein expression for survivin and XIAP, respectively, in the tumour cell lines. Pearson linear correlation coefficients r and their P-value are indicated.

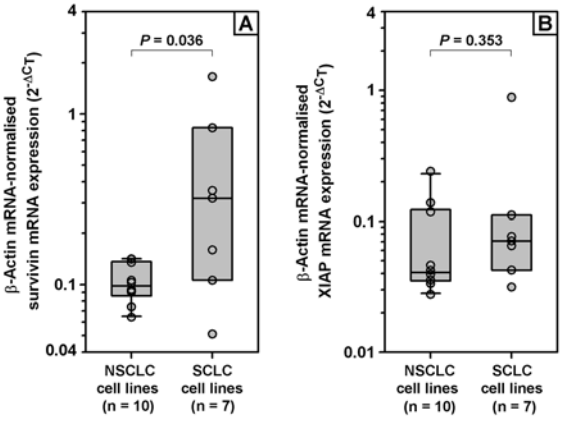

Figure 3. Comparison of survivin mRNA (A) and XIAP mRNA (B) expression, respectively, in non-small cell lung carcinoma (NSCLC) cell lines and small cell lung carcinoma (SCLC) cell lines. In the box plot, the upper and the lower boundary of the box and the line within the box indicate the 75th and 25th percentiles and the median, respectively. The error bars above and below the box indicate the 90th and 10th percentiles. Statistical difference (P) between the transcript expression levels was calculated using the Mann-Whitney test.

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

Tumour type	n	ß-actin mRNA-normalised expression of survivin mRNA ($2^{-\Delta C_T}$)[b]		Statistical difference (P) of survivin mRNA expression in Tu versus Lu[c]	Tu/Lu ratio of survivin mRNA expression[b]	Number of patients with Tu/Lu survivin mRNA expression ratio ≥2 and ≤0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC	150[a]	0.01423 (0.00072-0.20590)	0.00093 (0.000003-0.01499)	$2.7x10^{-43}$	16.2 (0.7-814.6)	142 (95%) and 0
SQCLC	69	0.01663[d] (0.00112-0.14865)	0.00102 (0.000047-0.00849)	$1.7x10^{-21}$	15.9 (1.1-537.4)	66 (96%) and 0
LAC	56	0.00837 (0.00072-0.11344)	0.00082 (0.000003-0.00671)	$1.5x10^{-16}$	15.0 (0.7-814.6)	52 (93%) and 0
LCLC	7	0.02977[d] (0.00286-0.20590)	0.00038 (0.000098-0.00613)	$1.16x10^{-3}$	29.0 (3.6-754.8)	7 (100%) and 0
SLC	4	0.01838 (0.01296-0.07081)	0.00127 (0.000676-0.00709)	$2.85x10^{-2}$	13.0 (2.3-104.7)	4 (100%) and 0
UNDIF	11	0.02538[d] (0.01024-0.10584)	0.00077 (0.000253-0.01499)	$2.35x10^{-4}$	40.5 (0.91-150.1)	10 (91%) and 0

[a]A total of 150 NSCLC patients was studied including 69 patients with squamous cell lung carcinoma (SQCLC), 56 patients with lung adenocarcinoma (LAC), 3 patients with SQCLC+LAC mixed-type tumours, 7 patients with large-cell lung carcinoma (LCLC), 4 patients with sarcomatoid lung carcinoma (SLC) and 11 patients with undifferentiated lung carcinoma (UNDIF). [b]Data are represented as the median with the range in parentheses. [c]The statistical difference of the ß-actin mRNA-normalised survivin mRNA expression in Tu versus Lu was calculated using the Mann-Whitney test. [d]The ß-actin mRNA-normalised survivin mRNA expression in SQCLC, LCLC and UNDIF was significantly higher than in LAC (P=0.0029, P=0.0187 and P=0.0035, respectively, Mann-Whitney test).

Table III. Immunometric analysis of survivin protein expression in non-small cell lung carcinomas 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)	$4.7x10^{-29}$	23.5 (1.1-720)	85 (97%) and 0
SQCLC	39	2.148 (0.107-15.901)	0.083 (0.015-0.325)	$3.2x10^{-13}$	27.2 (1.1-166.2)	37 (95%) and 0
LAC	38	2.189 (0.224-11.365)	0.079 (0.014-0.469)	$7.8x10^{-14}$	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 tumours, 2 patients with LCLC, 3 patients with SLC, and 3 patients with UNDIF. [b]Data are represented as the median with the range in parentheses. [c]Statistical difference of survivin protein expression in Tu versus Lu was calculated using the Mann-Whitney test.

of smoking and non-smoking LAC patients (P>0.07; Mann-Whitney test).

The expression of HBXIP mRNA, whose protein product functions as a co-factor for survivin in one of its apoptosis suppression mechanisms (3) in the studied NSCLC cell lines was strong ($2^{-\Delta C_T}$: median, 0.2747; range, 0.1081-0.6417) and significantly higher as compared to NSCLC tissues and lungs (Table V; $P=2.2x10^{-4}$ and $P=6.8x10^{-5}$, respectively; Mann-Whitney test). The expression of HBXIP mRNA in NSCLC tissues, except SLC, and matched lungs was not significantly different, and NSCLC tumours of different histopathological types expressed comparable levels of HBXIP mRNA (Table V). The number of patients with a tumour/lung HBXIP mRNA expression ratio >2 was relatively low in the group of all examined NSCLC patients (Table V), and there was no statistically significant impact of patient gender and smoking

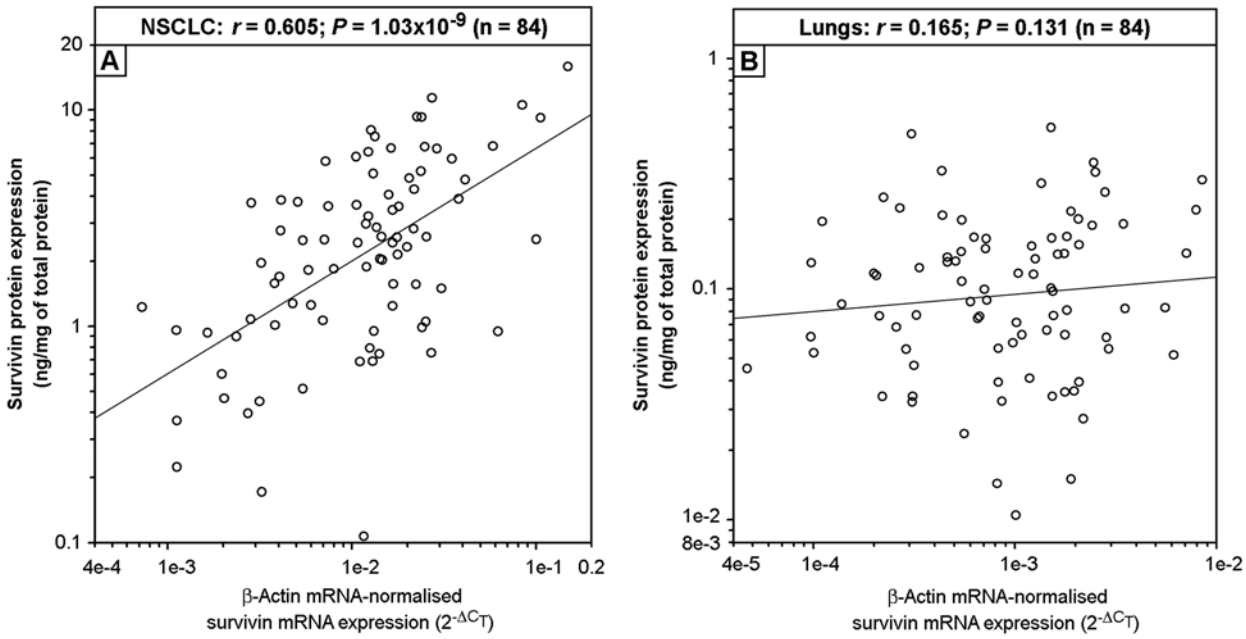

Figure 4. Correlation analysis of survivin mRNA and protein expression in non-small cell lung carcinoma (NSCLC) tissues (A) and lungs (B). Pearson linear correlation coefficients r and their P-value are indicated.

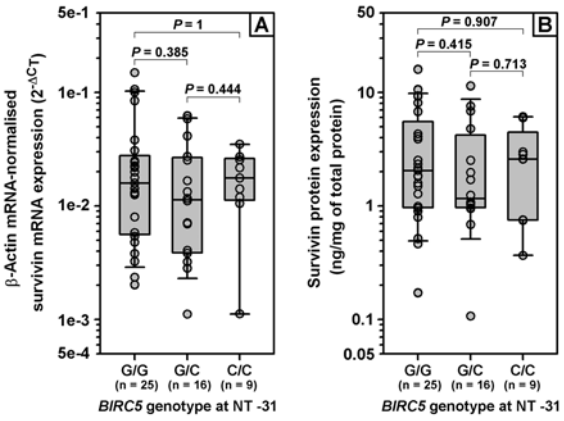

Figure 5. *BIRC5* promoter polymorphism at nucleotide -31 and the level of survivin mRNA (A) and survivin protein (B) expression in non-small cell lung carcinoma tissues. In the box plot, the upper and the lower boundary of the box and the line within the box indicate the 75th and 25th percentiles and the median, respectively. The error bars above and below the box indicate the 90th and 10th percentiles. Statistical difference (P) between the indicated genotype categories was calculated using the Mann-Whitney test.

habit, tumour grade and tumour stage on the expression of HBXIP mRNA in NSCLC tissues (P>0.11, Mann-Whitney test, data not shown).

The expression of XIAP mRNA in NSCLC tissues and matched lungs was not significantly different, and NSCLC tumours of different histopathological types showed comparable levels of XIAP mRNA (Table VI). In contrast, the expression of XIAP protein in NSCLC tissues, particularly in LACs, was significantly higher as compared to matched lungs (Table VII). Nevertheless, the number of patients with a tumour/lung XIAP mRNA and XIAP protein expression ratio >2 was relatively low in the groups of all examined NSCLC patients (Tables VI and VII). There was no correlation between XIAP mRNA and protein expression in the tumours and the lungs (Fig. 6). The expression of both XIAP mRNA and protein in the studied NSCLC tissues and NSCLC cell lines was not significantly different (P>0.78; Mann-Whitney test). Patient gender, tumour grade and tumour stage did not have any significant impact on the expression of XIAP mRNA and protein in NSCLC tissues. The tumours of smokers showed a significantly lower expression of XIAP protein, but not of mRNA, as compared to the tumours of non-smokers (Table VIII). However, when LACs were considered separately, the expression of XIAP protein in the tumours of smoking and non-smoking LAC patients was not statistically significant (P>0.12; Mann-Whitney test).

Although the molar levels of survivin and XIAP proteins were not significantly different in NSCLC cell lines (median/range, 538.5/214.5-942.0 and 682.9/202.6-1583.5 fmol/mg of total protein, respectively; P=0.34, Mann-Whitney test, n=10) the molar level of survivin protein was significantly lower than that of XIAP protein in NSCLC tissues (median/range, 145.4/6.5-970.1 and 599.8/4.3-1987.4 fmol/mg, respectively; $P=7.4x10^{-18}$, Mann-Whitney test, n=88). There was no correlation between the expression of survivin and XIAP proteins in NSCLC cell lines and NSCLC tissues or lungs.

Discussion

The results of the present study provide evidence that both survivin mRNA and protein are overexpressed in NSCLC cell lines and NSCLC tumours of different histopathological subtypes. Considering the major histopathological types of NSCLC and the degree of their differentiation, the expression of survivin mRNA was significantly higher in SQCLCs as compared to LACs, and significantly higher in the less-

Table IV. Impact of gender, smoking, tumour grade, and tumour stage on survivin mRNA and survivin protein expression in non-small cell lung carcinoma.

Category	n[a]	ß-actin mRNA-normalised expression of survivin mRNA ($2^{-\Delta C_T}$)[b]	Statistical difference (P)[c]	n[a]	Survivin protein expression (ng/mg of total protein)[b]	Statistical difference (P)[c]
Gender						
Men	110	0.01618 (0.00129-0.20590)	0.001	58	2.549 (0.107-15.901)	0.529
Women	40	0.00647 (0.00072-0.11344)		30	1.638 (0.224-11.365)	
Smoking						
Non-smokers	20	0.00625 (0.00072-0.05831)	0.011	13	1.276 (0.224-11.365)	0.371
Smokers	130	0.01595 (0.00111-0.62417)		75	2.465 (0.107-15.901)	
Tumour grade						
Grade 1+2	69	0.01060 (0.00072-0.11344)	0.007	33	2.333 (0.172-10.555)	0.298
Grade 3	54	0.01722 (0.00112-0.07081)		42	2.701 (0.107-15.901)	
Tumour stage						
Stage IA	21	0.00744 (0.00112-0.05872)		13	1.011 (0.172-5.944)	
Stage IB	62	0.01505 (0.00111-0.20590)	0.054	32	2.474 (0.107-11.365)	0.049
Stage II+III	63	0.01584 (0.00072-0.14865)	0.122	40	2.099 (0.197-15.901)	0.044

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

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

Tumour type	n	ß-actin mRNA-normalised expression of HBXIP mRNA ($2^{-\Delta C_T}$)[b]		Statistical difference (P) of HBXIP mRNA expression in Tu versus Lu[c]	Tu/Lu ratio of HBXIP mRNA expression[b]	Number of patients with Tu/Lu HBXIP mRNA expression ratio ≥2 and ≤0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC	68[a]	0.1001 (0.0018-2.0994)	0.1088 (0.0118-0.5743)	0.752	0.99 (0.01-5.35)	17 (26%) and 12 (19%)
SQCLC	30	0.0932 (0.0143-0.3737)	0.1304 (0.0198-0.3299)	0.307	0.97 (0.08-3.05)	5 (17%) and 8 (27%)
LAC	22	0.1189 (0.0018-0.6736)	0.1077 (0.0118-0.3392)	0.589	1.18 (0.01-5.35)	7 (32%) and 3 (14%)
LCLC	5	0.1476 (0.0638-2.0994)	0.1134 (0.0364-0.5743)	0.547	2.71 (0.73-3.65)	3 (60%) and 0
SLC	4	0.0417 (0.0326-0.0487)	0.0706 (0.0612-0.1134)	0.028	0.50 (0.40-0.79)	0 and 2 (50%)
UNDIF	7	0.1330 (0.0470-0.1550)	0.1096 (0.0369-0.2176)	1	1.27 (0.31-2.34)	2 (28%) and 1 (14%)

[a]A total of 68 NSCLC patients was studied including 30 patients with SQCLC, 22 patients with LAC, 5 patients with LCLC, 4 patients with SLC and 7 patients with UNDIF. [b]Data are represented as the median with the range in parentheses. [c]Statistical difference of the ß-actin mRNA-normalised expression of HBXIP mRNA in Tu versus Lu was calculated using the Mann-Whitney test.

differentiated than in the well-differentiated SQCLCs and LACs, respectively. These findings confirm previously reported results (56,57). Although the expression of survivin mRNA in stage IB and stage II+III tumours was higher than in stage IA tumours, this difference did not reach statistical significance. Nevertheless, we found that the expression of survivin protein was significantly upregulated in stage IB tumours and stage II+III tumours as compared to stage IA tumours. These immunometric data are consistent with previously reported immunohistochemical findings (56) and

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

Tumour type	n	ß-actin mRNA-normalised expression of XIAP mRNA ($2^{-\Delta C_T}$)[b]		Statistical difference (P) of XIAP mRNA expression in Tu versus Lu[c]	Tu/Lu ratio of XIAP mRNA expression[b]	Number of patients with Tu/Lu XIAP mRNA expression ratio ≥2 and ≤0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC	150[a]	0.06143 (0.00125-0.29321)	0.05892 (0.01017-0.30355)	0.659	0.90 (0.03-8.28)	12 (8%) and 21 (14%)
SQCLC	69	0.05954 (0.01075-0.16724)	0.05913 (0.01017-0.28127)	0.726	0.87 (0.32-8.28)	3 (4%) and 6 (9%)
LAC	56	0.06359 (0.00617-0.29321)	0.05852 (0.01332-0.30355)	0.960	0.94 (0.22-3.84)	8 (14%) and 10 (18%)
LCLC	7	0.06745 (0.03691-0.12763)	0.06381 (0.04419-0.10153)	0.898	1.10 (0.42-2.00)	1 (14%) and 1 (14%)
SLC	4	0.03470 (0.00982-0.05441)	0.06460 (0.02435-0.09408)	0.200	0.49 (0.38-0.76)	0 and 2 (50%)
UNDIF	11	0.05441 (0.02836-0.13870)	0.05913 (0.02521-0.20166)	0.947	0.98 (0.43-1.51)	0 and 1 (9%)

[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 are represented as the median with the range in parentheses. [c]Statistical difference of the ß-actin mRNA-normalised XIAP mRNA expression in Tu versus Lu was calculated using the Mann-Whitney test.

Table VII. Immunometric analysis of XIAP protein expression in non-small cell lung carcinomas 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 are respresented as the median with the range in parentheses. [c]Statistical difference of XIAP protein expression in Tu versus Lu was calculated using the Mann-Whitney test.

suggest that survivin may play a role in tumourigenesis and tumour growth of NSCLC. This notion is further substantiated by the immunohistochemical detection of increased survivin protein expression in premalignant stages of lung cancerogenesis including squamous metaplasia, dysplasia and atypical adenomatous hyperplasia (56,58-60).

The expression of survivin mRNA and protein showed a high positive correlation in NSCLC tumours, but there was lack of correlation of their expression in the lungs. The latter result points to the possibility that there may be an accelerated degradation of the weakly expressed survivin protein in normal lung cells, such as bronchial epithelia (59), which can be promoted by the XIAP•XAF1 protein complex (61). This view is supported by ubiquitous expression of XAF1, an antagonist of XIAP anti-caspase activity, in normal cells and tissues including lung (62) as well as by high expression of XIAP in normal cells including bronchial epithelia (63).

Since the occurrence of the C/C genotype at NT -31 in *BIRC5* promoter has been previously correlated with decreased survivin mRNA and protein expression in a variety of cancer cell lines (48), we examined the relationship between the *BIRC5* promoter genotype at NT -31 and the

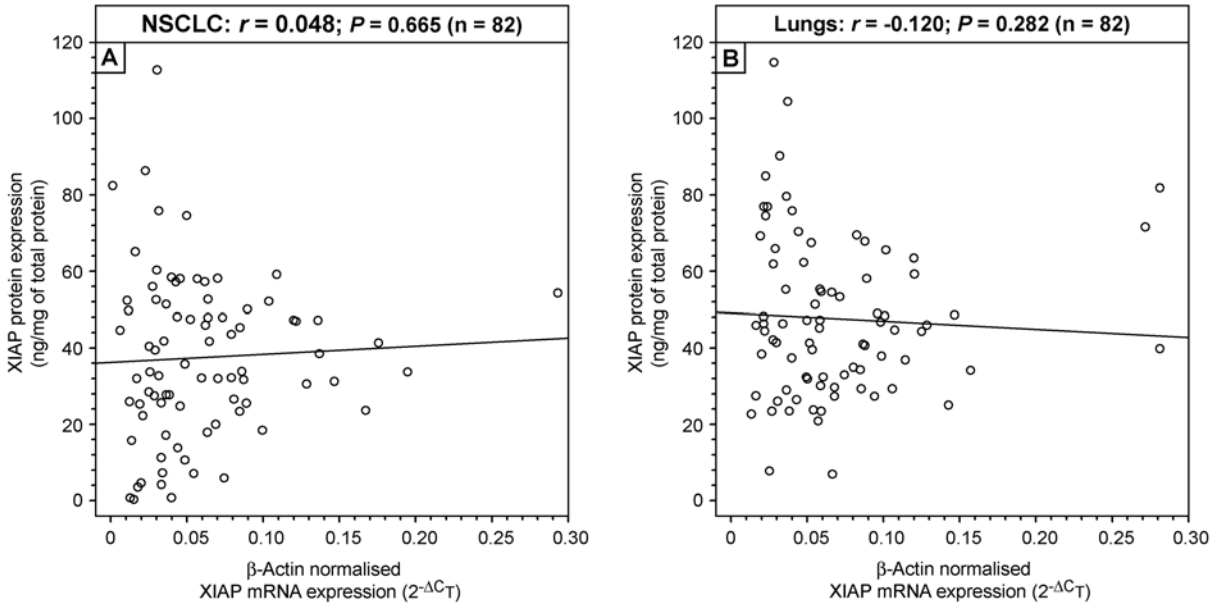

Figure 6. Correlation analysis of XIAP mRNA and protein expression in non-small cell lung carcinoma (NSCLC) tissues (A) and lungs (B). Pearson linear correlation coefficients r and their P-value are indicated.

Table VIII. Impact of gender, smoking, tumour grade, and tumour stage on XIAP mRNA and XIAP protein expression in non-small cell lung carcinoma.

Category	n[a]	ß-actin mRNA-normalised expression of XIAP mRNA ($2^{-\Delta C_T}$)[b]	Statistical difference (P)[c]	n[a]	XIAP protein expression (ng/mg of total protein)[b]	Statistical difference (P)[c]
Gender						
Men	110	0.06315 (0.00617-0.28519)	0.553	58	32.85 (0.71-86.27)	0.263
Women	40	0.05241 (0.00125-0.29321)		30	40.35 (0.24-112.70)	
Smoking						
Non-smokers	20	0.06381 (0.00125-0.28519)	0.746	13	49.68 (5.91-82.39)	0.009
Smokers	130	0.05913 (0.00617-0.29321)		75	32.84 (0.24-112.70)	
Tumour grade						
Grade 1+2	67	0.05872 (0.01075-0.28519)	0.871	36	32.51 (0.71-74.53)	0.205
Grade 3	54	0.06655 (0.00617-0.29321)		36	42.64 (0.25-86.27)	
Tumour stage						
Stage IA	21	0.05441 (0.00137-0.12158)		13	45.29 (5.91-112.70)	
Stage IB	62	0.06359 (0.00125-0.28519)	0.536	32	34.01 (3.50-86.27)	0.910
Stage II+III	63	0.06293 (0.01168-0.29321)	0.260	40	34.73 (0.24-74.53)	0.555

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

expression of survivin mRNA and protein in NSCLC cell lines and tissues. We found that the expression of both survivin mRNA and protein in the investigated tumour cell lines and tissues was not significantly affected by the *BIRC5* promoter genotype at NT -31. These results are in line with the recently reported immunohistochemical data for the expression of survivin in breast carcinomas (66). The overexpression of both survivin mRNA and protein in NSCLC cells and tissues may be primarily of the transcriptional origin, due to increased co-expression of several *BIRC5*-targeting transcription factors including E2F-1 (67), Stat3 (68), NF-κB (69), and HIF-1α (70). Moreover, the proteasomal degradation of survivin protein may be slowed down in NSCLC cells because of survivin ubiquitination inhibition, which can be caused by overexpression of cyclooxygenase-2 (71,72) and/or down-regulation of the survivin ubiquitinating XIAP•XAF1

complex (61) due to low or absent expression of XAF1 in NSCLC cells (62,64,65). The enhanced expression of survivin mRNA in SCLC cell lines as compared to NSCLC cell lines (55, and the present study) suggests that the transcriptional upregulation of *BIRC5* gene expression in NSCLC and SCLC cells may be different. Altogether, the high over-expression of the *BIRC5* gene in lung tumours offers the rationale for their apoptosis-based therapy that could involve the tumour-specific expression of recombinant DNA constructs consisting of a fusion of the *BIRC5* promoter to the coding sequence of an active apoptosis effector such as reverse caspases or granzyme B (73,74).

Survivin can form a protein complex with HBXIP which binds procaspase-9 and sequesters it from activation in the apoptosome (3). In this study, 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 is the only IAP which directly inhibits the activity of apoptotic caspases including caspase-9, -3 and -7 (9). Contrary to a previous report (75), the present study revealed that the expression of XIAP mRNA in NSCLC cell lines and tissues and lungs was not significantly different. However, we found a significantly increased level of XIAP protein in NSCLC tissues, particularly in LAC tumours, as compared to matched 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 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 (76-78). 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 (79) or interaction of XIAP with survivin (8), Notch receptor (80) or $p34^{SEI-1}$ protein (81). The upregulated expression of XIAP in cancer cells that occurs in response to DNA damage is due to the Che-1 protein- (82) and/or MDM2 protein-mediated (78) activation of XIAP NF-κB-dependent transcription and XIAP IRES-dependent translation, respectively.

Although the molar levels of survivin and XIAP proteins in NSCLC cell lines were not significantly different, the molar level of XIAP protein in NSCLC tumours was significantly higher than that of survivin protein. The latter difference may be due to slower expression of survivin protein rather than by its accelerated degradation since the rates of degradation of survivin and XIAP proteins in cancer cells seem to be similar (83).

The expression of XIAP mRNA and protein in NSCLC tumours did not show any significant relationship to clinico-pathological factors such as gender, tumour histology, differentiation and stage. These results are in line with previously reported data on XIAP expression in NSCLC tumours (75,84,85). Although the expression of XIAP and cIAPs in NSCLC tumours did not predict the response to classic chemotherapy in patients with advanced NSCLC (84), 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 (35,36,86,87) or sensitises them against apoptosis inducers such as non-steroidal anti-inflammatory drugs and TRAIL (88,89).

Acknowledgements

This work was supported by a grant (no. NR/7860-5) and an institutional research project (no. MZO 00064211) from the Ministry of Health, Czech Republic.

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