Down-regulated expression of apoptosis-associated genes APIP and UACA in non-small cell lung carcinoma

ERIKA MORAVCIKOVA[1,3], EVZEN KREPELA[1,3], JAN PROCHAZKA[1], ILONA ROUSALOVA[1], JAN CERMAK[2] and KAMILA BENKOVA[4]

[1]Laboratories of Molecular and Cell Biology and [2]Division of Surgery, Department of Pneumology and Thoracic Surgery, University Hospital Bulovka and 3rd Faculty of Medicine, [3]Institute of Biochemistry and Experimental Oncology, 1st Faculty of Medicine, Charles University in Prague; [4]Department of Pathology, University Hospital Bulovka, Prague, Czech Republic

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Abstract. The Apaf-1 interacting protein (APIP) and the uveal autoantigen with coiled coil domains and ankyrin repeats (UACA) belong to endogenous regulators of the apoptosome apparatus, but their role in tumourigenesis and progression of non-small cell lung carcinoma (NSCLC) is not known. Previous studies demonstrated that APIP inhibits the apoptosome-mediated procaspase-9 activation while UACA induces translocation of Apaf-1 from the cytoplasm into the nucleus. Here, we report for the first time that the expression of *APIP* and *UACA* genes is down-regulated on the level of both mRNA and protein in NSCLC cells and tumours. In particular, the expression of APIP protein was strikingly decreased and the expression of UACA mRNA and protein was frequently down-regulated in NSCLC tumours of different histopathological types. Moreover, stage IA NSCLC tumours showed significantly lower expression of UACA mRNA compared to higher stage tumours. The weak increase of both APIP and UACA mRNA levels in the 5-aza-2'-deoxycytidine-treated NSCLC cells indicates that mechanisms other than DNA methylation are involved in the regulation of *APIP* and *UACA* gene expression in these cancer cells. Taken together, the down-regulation of APIP and UACA expression suggests that the threshold to activate the apoptosome apparatus may be decreased in NSCLC cells due to the lack of APIP-mediated suppression and UACA-assisted Apaf-1 nuclear entry. Moreover, the loss of UACA-assisted Apaf-1 nuclear translocation may underlie the failure of DNA damage checkpoint activation in NSCLC cells leading to their genomic instability.

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

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Introduction

The apoptosome apparatus is a stress-induced cell death signalling platform that is assembled in the cytosol via the cytochrome-c (cyt-c)- and (d)ATP-mediated formation of an Apaf-1 heptameric complex, which recruits and activates the apoptosis initiator procaspase-9 (PC-9) (1-3). Both epigenetic and transcriptional factor-mediated regulation of Apaf-1 and PC-9 expression levels plays an important role in predisposition of cells to activate the apoptosome apparatus (4-8). However, the processes of apoptosome assembly and functioning in cancer cells are under a variety of negative and positive modes of post-translational regulation (9,10). There is evidence that the hypersensitivity of certain malignant neoplasms such as brain tumours and breast carcinomas, as opposed to their normal tissue counterparts, to the cyt-c-induced apoptosis is due to up-regulation of Apaf-1 and its regulator PHAPI, respectively, in cancer cells (11,12). On the other hand, deficient signalling in the apoptosome pathway, due to the lack of apoptosome core component expression or apoptosome dysfunction, contributes to tumourigenesis and progression of malignant neoplasms as well as to their chemo- and radioresistance (8,13-20).

The activation of apoptosome apparatus is often impaired in non-small cell lung carcinoma (NSCLC) cells and tissues (21,22). The molecular basis of apoptosome apparatus suppression in NSCLC is still unknown and there is evidence that it does not involve the segregatory binding of procaspase-9 to TUCAN (23) or direct inhibition of caspase-9 by XIAP (22,24). The Apaf-1 interacting protein (APIP/AIP) and the uveal autoantigen with coiled coil domains and ankyrin repeats (UACA)/nucling belong to the endogenous regulators of apoptosome apparatus (9,10), but their role in NSCLC tumourigenesis and progression is not known. APIP, which is a cytosolic protein, binds to the CARD domain of Apaf-1 and prevents PC-9 recruitment to the apoptosome (25,26). Moreover, during hypoxic conditions, APIP induces sustained activation of AKT and ERK1/2 kinases, which directly phosphorylate PC-9 to inhibit its activation in the apoptosome (27). UACA/nucling, showing both cytoplasmic and perinuclear/nuclear localization (28-30), interacts with Apaf-1 and induces its translocation into the nucleus upon proapoptotic stress (31). UACA also interacts with the nuclear

factor-κB (NF-κB) and prevents its entry into the nucleus thus reducing expression of the NF-κB-targeted genes (32).

To obtain insight into the role of APIP and UACA in NSCLC, we investigated their expression status in NSCLC cell lines and NSCLC tumours and matched lungs from surgically treated patients in relation to pathological and clinical data of the patients.

Patients and methods

Patients. One hundred and two patients with NSCLC tumours were investigated in this study (median age of 63 years, age range of 39-78 years; 72 men and 30 women; 89 smokers and 13 non-smokers). The patients did not receive any chemotherapy or radiotherapy prior to surgery. The surgical treatment applied to the patients included lung lobectomy, bilobectomy or pneumonectomy, and regional lymphnode dissection. The histopathological classification of lung tumours was carried out according to the World Health Organization criteria (33) and tumour staging was performed according to the new international pTNM system (34). The following NSCLC types were examined in the present study: squamous cell lung carcinoma (SQCLC; n=41), lung adenocarcinoma (LAC; n=36), 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). This study was approved by the local institutional ethics committee and was conducted in accordance with the Declaration of Helsinki. Signed informed consent was obtained from each patient before entry to the study.

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

Cell lines. NSCLC cell lines were obtained from the following sources: CALU-1, SKMES-1, A549, SKLU-1 and COR-L23 from the European Collection of Cell Cultures, Salisbury, UK; LXF-289, COLO-699 and LCLC-103H from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; NCI-H520 and NCI-H1299 from the American Type Culture Collection (ATCC), Rockville, MD, USA. The small cell lung carcinoma (SCLC) cell lines NCI-H69, NCI-H146, NCI-H209, NCI-H345, NCI-H378, NCI-H82 and NCI-H446 were obtained from ATCC. Culture media and their supplements were from Sigma (St. Louis, MO, USA). The NSCLC cell lines were cultured in humidified atmosphere of air with 5% CO_2 at 37˚C in the Eagle's minimum essential medium supplemented with 5% fetal 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 (the EMEM-S medium). After reaching confluency, the adherent cells were harvested by scraping into a lysis solution for the isolation of total RNA or into the Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution for preparation of protein extracts (see below).

The SCLC cell lines were grown in humidified atmosphere of air with 5% CO_2 at 37˚C in RPMI-1640 medium supplemented with 10% fetal 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. These cells, growing loosely in suspension, were harvested by centrifugation at 240 g and 4˚C for 10 min.

The effect of 5-aza-2'-deoxycytidine (ADC; Sigma) on APIP and UACA mRNAs expression in the NSCLC cell lines was studied as follows. The cells were seeded in the EMEM-S medium which was changed the next day for the fresh medium without (control cell cultures) or with 10 μM of ADC. The cells were then cultured for 72 h with the media change after the first 48 h. After the treatment, the adherent cells were harvested for total RNA isolation as described above.

Isolation and quantification of total RNA. Total RNA was isolated from cells and tissues using the TRIzol Reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. All isolated RNA samples had an $A_{260\ nm}/A_{280\ nm}$ ratio >1.8, as measured by spectrophotometric analysis in 10 mM Tris/HCl buffer, pH 7.5. The concentration of total RNA was determined by a fluorometric assay using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA) and the manufacturer's protocol.

Real-time RT-PCR analysis. The sequences and final concentrations of the oligonucleotide primers and TaqMan probes used in real-time RT-PCR assays of expression of the investigated transcripts are indicated in Table I. The primers and probes were designed using the program Primer Express (Applied Biosystems, Foster City, CA, USA) and were synthesized at Microsynth (Balgach, Switzerland) and Applied Biosystems (Warrington, UK), respectively. The expression of APIP, UACA and β-actin (an internal reference transcript) mRNAs was quantitated by a coupled real-time RT-PCR assay. The RT-PCR reaction mixtures of a total volume of 50 μl contained 25 μl of SuperScript Reaction Mix (a buffer with 6 mM $MgSO_4$ and 400 nM of each dGTP, dCTP, dATP and dTTP) and 1 μl of SuperScript III Reverse Transcriptase/Platinum *Taq* DNA polymerase mix (both mixes were from Platinum Quantitative RT-PCR SuperScript III One-Step System, Invitrogen), the respective primers and TaqMan probe at the indicated final concentrations (Table I), 40 U of an RNase inhibitor RNaseOUT (Invitrogen), and 200 ng of total RNA. The final concentrations of primers and probes adopted for real-time RT-PCR quantification of each indicated transcript were determined in optimisation experiments. The real-time RT-PCR assays were run in duplicate in MicroAmp Optical 96-well reaction plates on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The reverse transcription was carried out at 58˚C for 30 min and the subsequent PCR amplification included a hot start at 95˚C for 5 min, 45 cycles of denaturation at 95˚C for 15 sec and of annealing/extension at 58˚C for 1 min. The threshold cycle (C_T) values of the amplification reactions, represented by the plots of background-subtracted fluorescence intensity (ΔFU) of the reporter dye (6-FAM or VIC) against PCR cycle number, were determined with the Sequence Detection System software (Applied Biosystems). The statistical difference of the β-actin mRNA-normalized target transcript expression in tumours and lungs was calculated from the linearised ΔC_T data (i.e., $2^{-\Delta C_T}$)

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 (nM)
APIP	NM_015957	Forward: 5'-GGAACGAATTCAGCCTGAAG-3'	400
		Reverse: 5'-CACCTGCTCCTCTCATTGTGT-3'	400
		TaqMan: 5'-(6-FAM)AAGGACATAAGTGGACCTTCGCCATCG(TAMRA)-3'	200
UACA	NM_006402	Forward: 5'-CATCCTTATACATGGAGTTGATATTACAA-3'	400
		Reverser: 5'-TGTCCGCCCGTCTACATC-3'	400
		TaqMan: 5'-(6-FAM)CTGCACTTCACGATGCCGCAATG(TAMRA)-3'	200
β-actin	NM_001101	Forward: 5'-CTGGCACCCAGCACAATG-3'	200
		Reverse: 5'-GGGCCGGACTCGTCATAC-3'	200
		TaqMan: 5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3'	200

and the tumour/lung ratio of the β-actin mRNA-normalized target transcript expression was calculated by means of the $2^{-\Delta\Delta C_T}$ method (35).

Preparation of protein extracts and total protein determination. Adherent NSCLC cells growing in monolayer were scraped into the Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and sedimented by centrifugation at 240 g and 4˚C for 10 min. The harvested cells were disrupted by sonication, at 0-4˚C, in phosphate buffered saline, pH 7.2, containing 0.5% Triton X-100 and a mixture of proteinase inhibitors including 1 mM Na_2EDTA, 50 μM E-64, 200 μM AEBSF and 25 μM pepstatin. The cell homogenates were centrifuged at 40,000 x g and 4˚C for 30 min. The collected supernatants, i.e., protein extracts, were stored in small aliquots at -78˚C until analysis. Total protein concentration was determined by the bicinchoninic acid assay using bovine serum albumin (BSA) as a standard (36).

Western blot analysis. The expression of APIP and UACA proteins in NSCLC cells and tissues and lungs was analysed by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using 16.5% T/3% C and 8% T/3% C polyacrylamide gels, respectively, and immunoblotting with specific antibodies. Samples of protein extracts from cells and tissues were denatured by boiling 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 proteins (100 μg per gel lane) were then separated by SDS-PAGE using the Tris-Tricine-SDS buffer system (37). The Precision Plus Protein Prestained Standards (Bio-Rad, Hercules, CA, USA) were run in parallel to the samples. The separated proteins were electrotransferred onto Hybond P membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) using a transfer buffer 48 mM Tris, 39 mM glycine, 1.3 mM SDS, and 20 v/v % methanol, pH 9.2. APIP and UACA proteins were visualised by an immunodetecting procedure coupled to an enhanced chemiluminescence generating system. The primary antibodies against APIP and UACA were APIP (D-20), the goat polyclonal IgG (Cat. no. sc-66318), and Nucling (E-12), the rabbit polyclonal IgG (cat. no. sc-135510), respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies were the rabbit anti-goat IgG-HRP peroxidase conjugate (Sigma) and the goat anti-rabbit IgG-HRP peroxidase conjugate (Sigma), respectively. The immunoblotting procedure was done at room temperature with the following steps: blocking with 5% blotting grade blocker (BGB; Bio-Rad) and 1% bovine serum albumin (BSA) in TBST buffer (20 mM Tris/HCl, 100 mM NaCl, and 0.1% Tween-20, pH 7.6) for 1 h, incubation with the primary antibody (at 1 μg/ml in 1% BGB and 1% BSA in TBST buffer) for 2 h, extensive washing with TBST buffer, incubation with the secondary HRP-conjugated antibody (at a dilution of 1:10,000 in 1% BGB and 1% BSA in TBST buffer) for 1 h, extensive washing with TBST 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 APIP and UACA protein bands in the film immunograms was evaluated with the 1D Image Analysis Software (Eastman Kodak).

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

Results

To analyse the expression status of APIP and UACA mRNAs in NSCLC cells and tissues and lungs we quantitated the level of these transcripts and β-actin mRNA (an endogenous reference transcript) using coupled real-time RT-PCR. A set of PCR amplification plots which is shown in Fig. 1 illustrates decreased expression of APIP and UACA mRNAs in NSCLC tissues as compared to matched lungs, but comparable expression of β-actin mRNA in tumour-lung matched pairs. Although the expression of β-actin mRNA (calculated as 2^{-C_T} data) was not significantly different in LCLC, SLC and UNDIF tumours and matched lungs ($P>0.1$, Mann-Whitney U test), it was slightly but significantly higher in SQCLC and

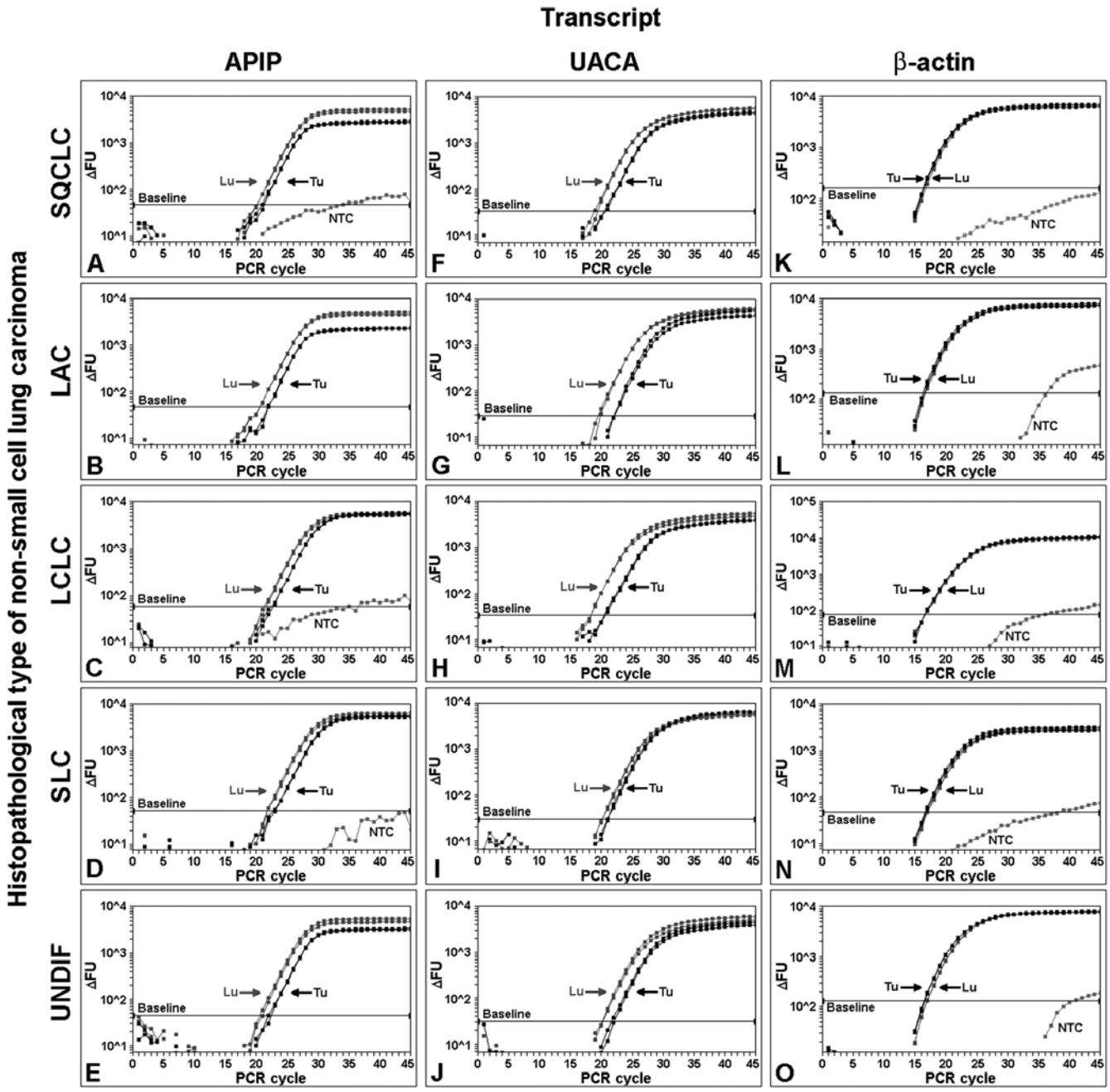

Figure 1. Real-time RT-PCR quantitation of expression of APIP, UACA and β-actin mRNAs in various histopathological types of non-small cell lung carcinoma and matched lungs. The set of PCR amplification plots, representing the coupled real-time RT-PCR assays with an input of 200 ng of total RNA and running in duplicate, shows the expression of APIP (A-E), UACA (F-J) and β-actin (K-O) 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).

LAC tumours as compared to matched lungs ($P=3.87x10^{-6}$ and $P=5.22x10^{-6}$, respectively, Mann-Whitney U test).

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 (Figs. 2 and 3). In the case of APIP, but not UACA, the expression of mRNA and protein showed a significant positive Pearson linear correlation (r=0.854, P=0.0069). Different histopathological types of NSCLC tumours and matched lungs also expressed both APIP and UACA mRNAs and proteins (Figs. 1, 2 and 3). However, as compared to matched lungs, the expression of APIP and UACA mRNAs and proteins in the tumours was markedly decreased (Figs. 1, 2 and 3).

We also analysed the expression of APIP and UACA mRNAs in a series of SCLC cell lines. Although the level of APIP mRNA expression in NSCLC and SCLC cell lines was comparable (Fig. 4), the level of UACA mRNA expression was significantly higher in NSCLC cells than in SCLC cells (Fig. 4). The expression of β-actin mRNA (2^{-C_T} data) in NSCLC and SCLC cell lines was not significantly different (P>0.1, Mann-Whitney U test).

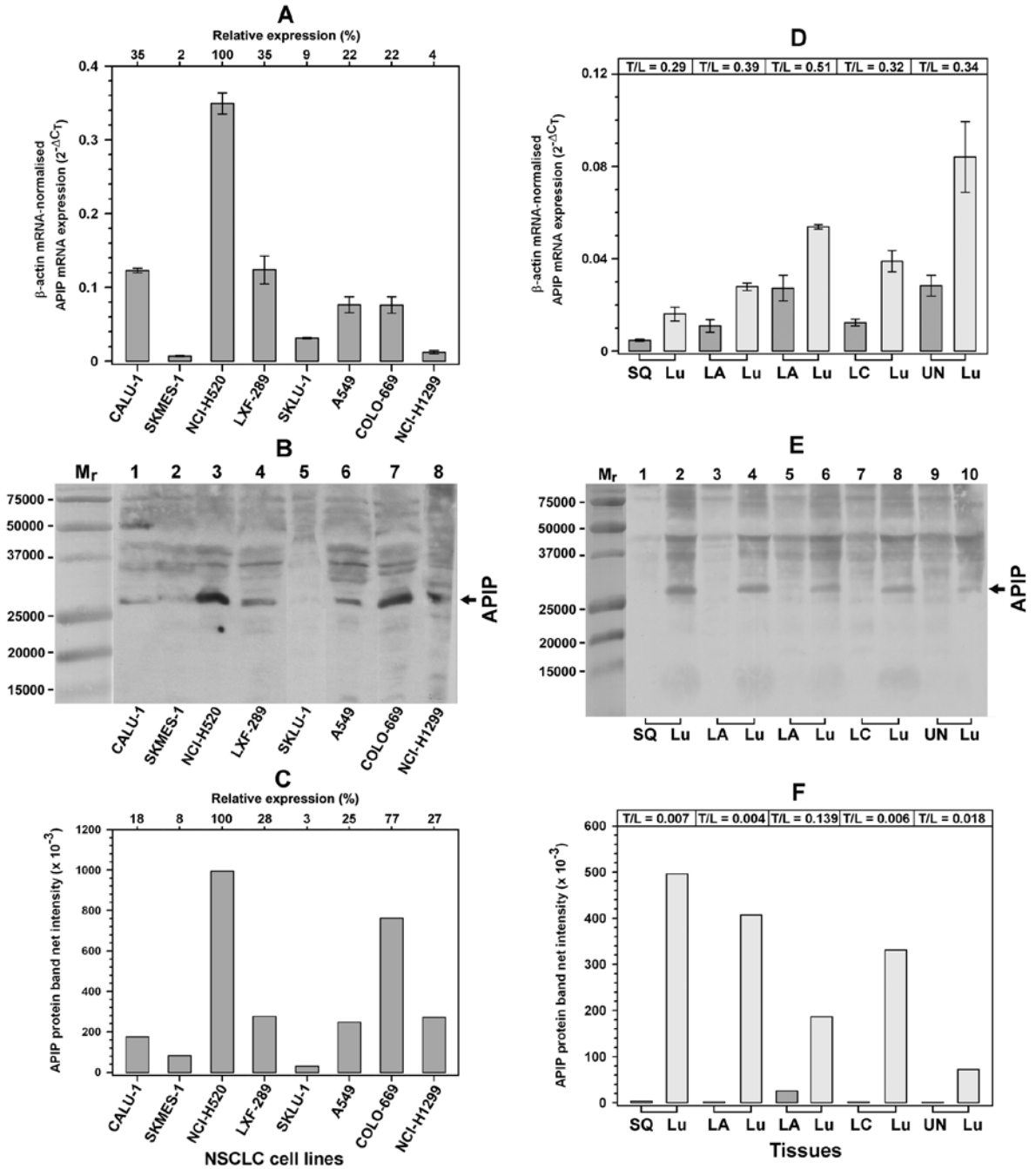

Figure 2. Analysis of APIP expression in non-small cell lung carcinoma cell lines and tumours and lungs. (A) Expression of APIP mRNA in the cell lines as quantitated by real-time RT-PCR. Data are indicated as the mean ± SEM from three independent experiments. Relative expression of APIP mRNA is shown at the top of the graph. (B) Expression of APIP protein (an apparent M_r of 28,000) in the cell lines as analysed by SDS-PAGE and immunoblotting. One representative experiment of three independent ones is shown. (C) Relative expression of APIP protein in the cell lines as evaluated by image analysis of the immunogram shown (B). (D) Decreased expression of APIP mRNA in five different NSCLC tumours as compared to matched lungs. Data are indicated as the mean ± SEM from real-time RT-PCR assays done in triplicate. The tumour/lung (T/L) ratio of APIP mRNA expression is shown at the top of the graph. The histopathological types of NSCLC tumours were squamous cell lung carcinoma (SQ), lung adenocarcinoma (LA), large cell lung carcinoma (LC), and undifferentiated lung carcinoma (UN). (E) Strikingly decreased expression of APIP protein [an apparent M_r of 28,000) in NSCLC tumours as compared to matched lungs, as analysed by SDS-PAGE and immunoblotting (the same tumour-lung matched pairs as in (D) were examined]. One representative experiment of three independent ones is shown. (F) Image analysis of the immunogram shown (E). The T/L ratio for APIP protein expression is shown at the top of the graph. The expression of APIP protein in the tumours is substantially lower as compared to matched lungs (P=0.0079, Mann-Whitney U test).

To test whether DNA methylation is involved in the transcriptional regulation of APIP and UACA expression in NSCLC cell lines, we cultured the tumour cells in the presence and the absence of 5-aza-2'-deoxycytidine (ADC), a DNA demethylating agent capable of up-regulating expression of many genes in various cancer cells (38-40). Although we detected statistically significant increase of APIP and UACA mRNAs expression in several NSCLC cell lines cultured in the presence of ADC (Fig. 5), the ADC-induced up-regulation of transcript expression higher than 2-fold was observed only for UACA mRNA in CALU-1 cells (a 3.9-fold increase; Fig. 5B). There was no significant difference between the β-actin mRNA expression

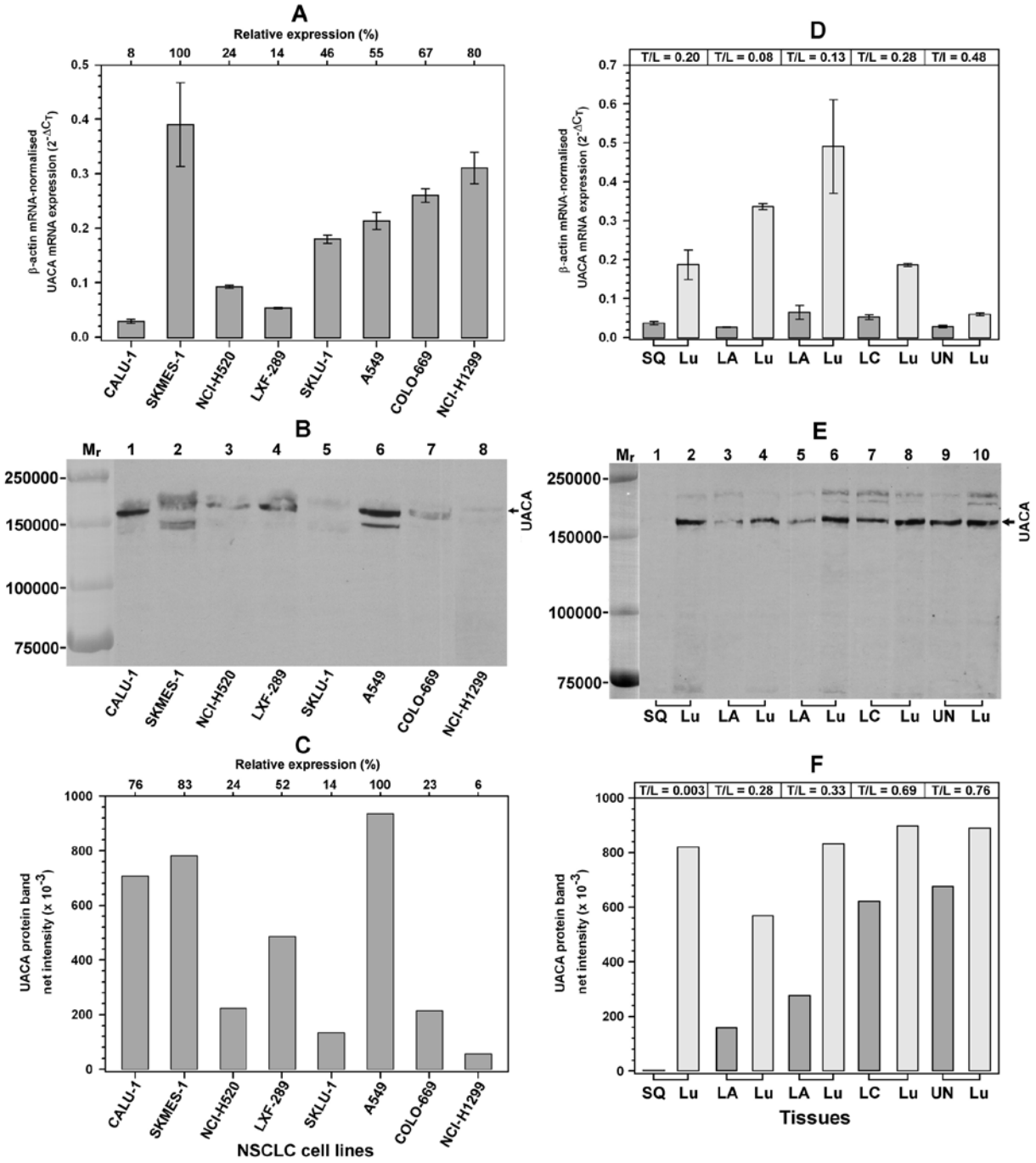

Figure 3. Analysis of UACA expression in non-small cell lung carcinoma cell lines and tumours and lungs. (A) Expression of UACA mRNA in the cell lines as quantitated by real-time RT-PCR. Data are indicated as the mean ± SEM from three independent experiments. Relative expression of UACA mRNA is shown at the top of the graph. (B) Expression of UACA protein (an apparent M_r of 170,000-180,000) in the cell lines as analysed by SDS-PAGE and immunoblotting. One representative experiment of three independent ones is shown. (C) Relative expression of UACA protein in the cell lines as evaluated by image analysis of the immunogram shown (B). (D) Decreased expression of UACA mRNA in five different NSCLC tumours as compared to matched lungs. Data indicated as the mean ± SEM from real-time RT-PCR assays done in triplicate. The tumour/lung (T/L) ratio of UACA mRNA expression is shown at the top of the graph. The examined tumour-lung matched pairs were the same as indicated in Fig. 2. (E) Decreased expression of UACA protein (an apparent M_r of 170,000) in NSCLC tumours as compared to matched lungs, as analysed by SDS-PAGE and immunoblotting [the same tumour-lung matched pairs as in (D) were examined]. One representative experiment of three independent ones is shown. (F) Image analysis of the immunogram shown (E). The T/L ratio for UACA protein expression is shown at the top of the graph. The expression of UACA protein in the tumours is significantly lower as compared to matched lungs (P=0.013, Mann-Whitney U test).

(2^{-C_T} data) in the ADC-treated and -untreated NSCLC cell lines (P>0.065, t-test).

In order to reveal the relationship between APIP and UACA mRNA expression in NSCLC tumours and the clinicopathological data of NSCLC patients, we studied the expression status of these transcripts in the tumours and matched lungs from surgically treated NSCLC patients. All examined NSCLC tumours and lung tissues showed the expression of APIP mRNA, but the level of APIP mRNA in the tumours was 2-fold or more down-regulated in one third of studied NSCLC patients (Table II). In SQCLC and LAC tumours, the expression of APIP mRNA was significantly lower as compared to matched lungs, while in

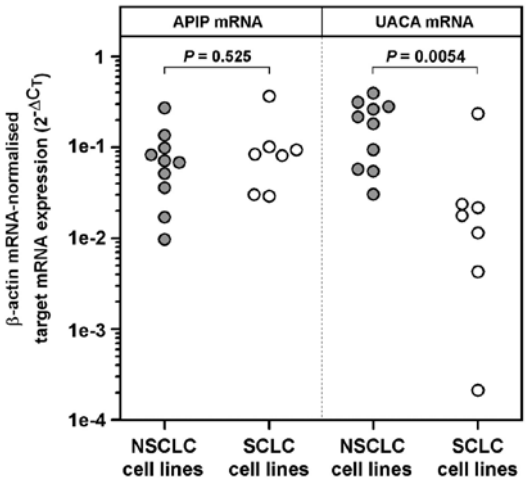

Figure 4. Comparison of APIP mRNA (A) and UACA mRNA (B) expression, respectively, in non-small cell lung carcinoma (NSCLC) cell lines and small cell lung carcinoma (SCLC) cell lines. Statistical difference (P) between the transcripts expression levels was calculated by Mann-Whitney U test.

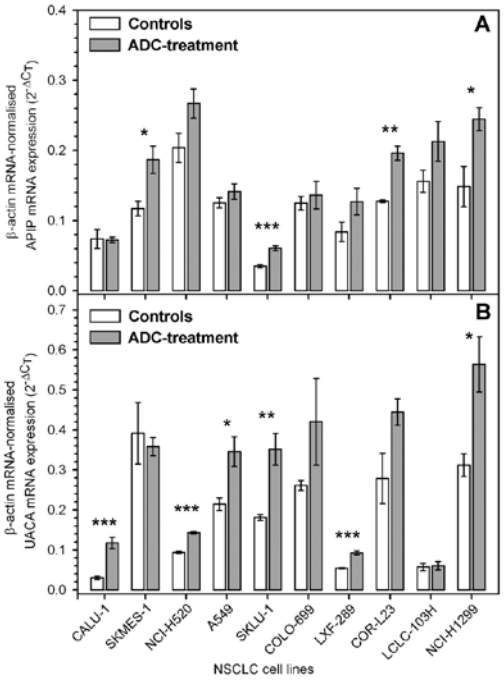

Figure 5. Effect of 5-aza-2'-deoxycytidine (ADC) on the expression of APIP mRNA (A) and UACA mRNA (B) in cultured NSCLC cell lines. Data are indicated as the mean ± SEM from three independent experiments. The asterisks indicate statistically significant up-regulation of transcripts expression in the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

other NSCLC tumour types (LCLCs, SLCs and UNDIFs) and matched lungs the expression of APIP mRNA was not significantly different (Table II). UNDIF tumours showed significantly higher expression of APIP mRNA compared to SQCLCs and

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

Tumour type	n	β-actin mRNA-normalised expression of APIP mRNA ($2^{-\Delta C_T}$)[b]		Statistical difference (P) of APIP mRNA expression in Tu versus Lu[c]	Tu/Lu ratio of APIP mRNA expression[b]	No. of patients with Tu/Lu APIP mRNA expression ratio ≥2 and ≤0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC (all types)	102[a]	0.0302 (0.0046-0.1119)	0.0467 (0.0145-0.6974)	$6.9x10^{-5}$	0.64 (0.07-2.13)	1 (1%) and 34 (33%)
SQCLC	41	0.0312 (0.0046-0.1066)	0.0474 (0.0158-0.6974)	$1.6x10^{-3}$	0.63 (0.07-1.34)	0 and 14 (34%)
LAC	36	0.0268 (0.0088-0.1119)	0.0467 (0.0187-0.2089)	$4.5x10^{-3}$	0.54 0.28-2.13)	1 (3%) and 15 (42%)
LCLC	7	0.0372 (0.0123-0.0670)	0.0387 (0.0257-0.0713)	0.382	0.91 (0.32-1.19)	0 and 1 (14%)
SLC	4	0.0451 (0.0130-0.0872)	0.0433 (0.0319-0.0656)	0.885	0.90 (0.36-1.73)	0 and 1 (25%)
UNDIF	11	0.0612 (0.0046-0.1119)	0.0529 (0.0145-0.2624)	0.693	1.16 (0.32-1.99)	0 and 3 (27%)

[a]A total of 102 NSCLC patients was studied including 41 patients with squamous cell lung carcinoma (SQCLC), 36 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]Statistical difference of the β-actin mRNA-normalised APIP mRNA expression in Tu versus Lu was calculated by Mann-Whitney U test.

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

Tumour type	n	β-actin mRNA-normalised expression of UACA mRNA ($2^{-\Delta C_T}$)[b]		Statistical difference (P) of UACA mRNA expression in Tu versus Lu[c]	Tu/Lu ratio of UACA mRNA expression[b]	No. of patients with Tu/Lu UACA mRNA expression ratio ≥2 and ≤0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC (all types)	102[a]	0.0494 (0.0137-0.2872)	0.1411 (0.0033-0.9330)	$1.4x10^{-17}$	0.40 (0.08-8.0)	2 (2%) and 72 (70%)
SQCLC	41	0.0480 (0.0172-0.2483)	0.1340 (0.0124-0.9330)	$2.8x10^{-7}$	0.40 (0.13-2.1)	1 (2%) and 29 (71%)
LAC	36	0.0467 (0.0137-0.2872)	0.1446 (0.0033-0.5249)	$6.4x10^{-7}$	0.35 (0.08-8.0)	1 (3%) and 25 (69%)
LCLC	7	0.0526 (0.0407-0.1088)	0.1869 (0.0718-0.3415)	$4.9x10^{-3}$	0.31 (0.19-0.74)	0 and 5 (71%)
SLC	4	0.0695 (0.0226-0.0988)	0.1139 (0.0515-0.1719)	0.470	0.45 (0.38-1.44)	0 and 3 (75%)
UNDIF	11	0.0608 (0.0257-0.2132)	0.1285 (0.0599-0.4965)	0.018	0.47 (0.31-1.58)	0 and 8 (73%)

[a]A total of 102 NSCLC patients was studied including 41 pacients with SQCLC, 36 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 β-actin mRNA-normalised UACA mRNA expression in Tu versus Lu was calculated by Mann-Whitney U test.

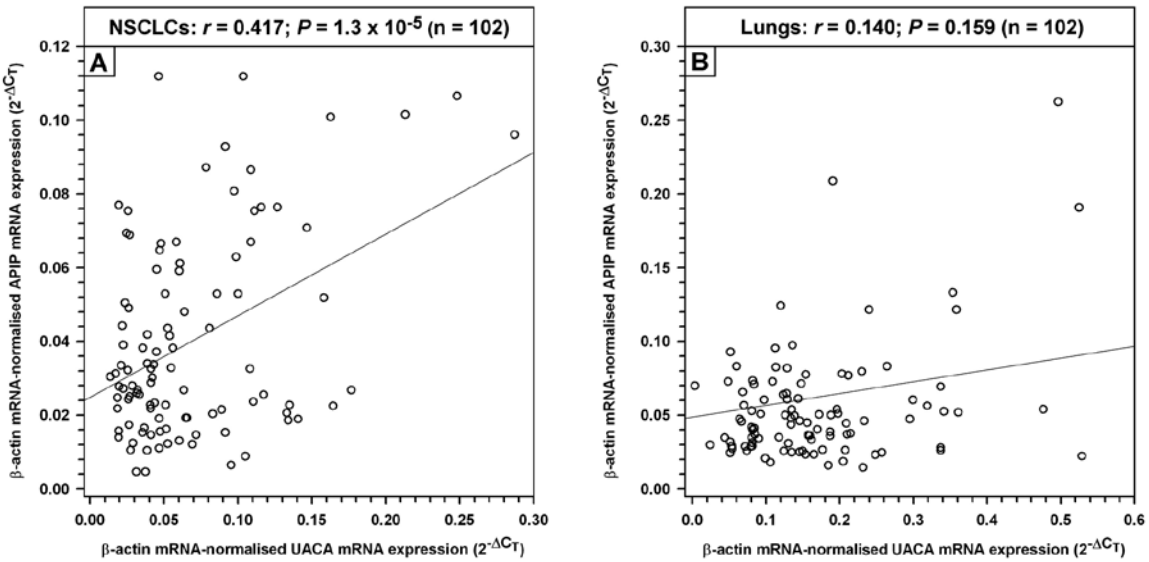

Figure 6. Correlation analysis of APIP and UACA mRNA expression in NSCLC tumours and lungs. The Pearson linear correlation coefficient *r* and its P-value are indicated.

LACs (P=0.023 and 0.015, respectively, Mann-Whithney U test). All investigated NSCLC tumours and lung tissues also expressed UACA mRNA, but expression of this transcript in the tumours was 2-fold or more down-regulated in a high proportion (70%) of examined NSCLC patients (Table III). As compared to matched lungs, the expression of UACA mRNA was significantly lower in different histopathological types of NSCLC tumours including SQCLCs, LACs, LCLCs and UNDIFs (Table III). There was no significant difference between the levels of UACA mRNA expression in different NSCLC tumour types (P>0.27). The

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

Category	n[a]	β-actin mRNA-normalised expression of APIP ($2^{-\Delta C_T}$)[b]	Statistical difference (P)[c]
Gender			
Men	72	0.0327 (0.0042-0.1321)	0.577
Women	30	0.0269 (0.0087-0.1216)	
Smoking			
Non-smokers	13	0.0302 (0.0173-0.0708)	0.523
Smokers	89	0.0304 (0.0046-0.1119)	
Tumour grade			
Grade 1+2	33	0.0259 (0.0064-0.1119)	0.847
Grade 3	39	0.0268 (0.0046-0.1008)	
Tumour stage			
Stage IA	15	0.0280 (0.0123-0.0866)	
Stage IB	42	0.0333 (0.0046-0.1119)	0.379
Stage II+III	41	0.0268 (0.0104-0.1119)	0.579

[a]The number 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 Mann-Whitney U test. The levels of APIP mRNA expression in stage IB tumours and stage II+III tumours were compared, respectively, with those in stage IA tumours.

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

Category	n[a]	β-actin mRNA-normalised expression of UACA mRNA ($2^{-\Delta C_T}$)[b]	Statistical difference (P)[c]
Gender			
Men	72	0.0475 (0.0137-0.1406)	0.436
Women	30	0.0492 (0.0172-0.2872)	
Smoking			
Non-smokers	13	0.0421 (0.0186-0.2872)	0.485
Smokers	89	0.0508 (0.0137-0.2483)	
Tumour grade			
Grade 1+2	33	0.0412 (0.0186-0.2483)	0.075
Grade 3	39	0.0559 (0.0172-0.2872)	
Tumour stage			
Stage IA	15	0.0317 (0.0137-0.1088)	
Stage IB	42	0.0531 (0.0194-0.2132)	0.025
Stage II+III	41	0.0518 (0.0186-0.2872)	0.014

[a]The number 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 Mann-Whitney U test. The levels of UACA mRNA expression in stage IB tumours and stage II+III tumours were compared, respectively, with those in stage IA tumours.

expression of APIP and UACA mRNAs showed a weak positive linear correlation in NSCLC tumours but not in the lungs (Fig. 6). Such correlation was observed also in certain histopathological types of NSCLC including SQCLC (r=0.357, P=0.022), LCLC (r=0.848, P=0.015) and UNDIF (r=0.624, P=0.040). Patients gender, smoking status and tumour grade did not significantly affect the expression of APIP and UACA mRNAs (Tables IV and V). Although the tumour stage did not have significant impact on the expression of APIP mRNA in NSCLC tissues (Table IV), the expression of UACA mRNA was significantly lower in stage IA tumours as compared to stage IB tumours and higher stage tumours (Table V). 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%).

Discussion

In the present study, we demonstrated that *APIP* and *UACA* genes are expressed at both mRNA and protein levels in NSCLC cell lines and NSCLC tumours and lungs. 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. Moreover, although the expression of APIP mRNA in NSCLC and SCLC cells was comparable, the expression of UACA mRNA in SCLC cells was significantly lower as compared to NSCLC cells. These results suggest that *UACA* gene might belong to the category of genes in which down-regulation brings survival advantage to cancer cells. This is in line with recent experimental data showing that nucling-knockout mice are not only resistant to the neurotoxin-induced apoptosis (41) but also frequently develop hepatocellular carcinoma (42). The lower level of UACA mRNA in stage IA tumours as compared to higher stage tumours suggests that the down-regulation of *UACA* gene expression is of particular importance during the earlier period of NSCLC development. The weak increase of expression of both APIP and UACA mRNAs in the 5-aza-2'-deoxycytidine (ADC)-treated NSCLC cell lines indicates that mechanisms other than DNA methylation are involved in regulation of *APIP* and *UACA* genes expression in these cancer cells. Contrary to the weak up-regulation of APIP and UACA mRNA expression, the expression of serpinB9 mRNA was strongly induced in several ADC-treated NSCLC cell lines (Rousalova *et al*, unpublished data).

It has been reported that ischemia/hypoxia up-regulates the expression of APIP in some tissues and tumour cells (25-27). Although there is evidence that the microenvironment of NSCLC tumours contains many ischemic regions characterized by hypoxia and acidosis (43), 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 the tongue and larynx where its expression is up-regulated and associates with increased copy number of the *APIP* gene (44).

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 (45). Interestingly, the Apaf-1^{Nuc} group of NSCLC patients had significantly better overall survival rate as compared to the Apaf-1^{Cyt} group (45). Recently, a non-apoptotic role for the intranuclear Apaf-1 in the DNA damage checkpoint activation has been demonstrated (46). As increased expression of UACA/nucling has been implicated in promoting Apaf-1 nuclear translocation after proapoptotic stress (31), 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 (32). 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 cell survival (47,48).

Taken together, the results of the present study provide evidence that the expression of *APIP* and *UACA* genes is down-regulated in NSCLC cells and tissues. The down-regulation of *UACA* gene is more pronounced and highly frequent in stage IA tumours as compared to higher stage tumours. The absence of strong induction of APIP and UACA mRNAs expression in the 5-aza-2'-deoxycytidine-treated NSCLC cell lines indicates that DNA methylation is not responsible for the regulation of *APIP* and *UACA* genes expression in these cancer cells. It can be expected that the down-regulation of APIP and UACA expression may decrease the threshold of NSCLC cells to the apoptosome apparatus activation due to the lack of APIP-mediated suppression and UACA-assisted Apaf-1 nuclear entry. Moreover, the loss of UACA-assisted Apaf-1 nuclear translocation may underlie the failure of DNA damage checkpoint activation in NSCLC cells leading to their genomic instability, which can causally contribute to development and progression of NSCLC tumours.

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