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Cancer-associated differences in the acetylcholinesterase activity in bronchial aspirates of lung cancer patients.

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Running title: non-neuronal cholinergic system in lung cancer

SUMMARY

In non-neuronal contexts, acetylcholine is thought to be involved in the regulation of vital cell functions such as proliferation, differentiation, apoptosis and cell-cell interaction. In airways most cells express the non-neuronal cholinergic system each containing a specific set of components required for synthesis, signal transduction and acetylcholine hydrolysis. Our aim was determine the expression of cholinergic system components in bronchial aspirates from controls and lung cancer patients. We conducted an analysis of cholinergic components in stored soluble and cellular fraction of bronchial aspirates from non-cancerous patients and diagnosed lung cancer patients. This study presents data proving that the fluid secreted by human lung cells contains enough acetylcholinesterase activity to control acetylcholine level. Thus, these data demonstrate that: (a) Acetylcholinesterase activity is significantly lower in aspirates from squamous cell carcinomas; (b) The molecular distribution of acetylcholinesterase in both bronchial cells and fluids consisted of amphiphilic monomers and dimers; (c) choline acetyltransferase, nicotinic receptors and cholinesterases are expressed in cultured human lung cells, as demonstrated by RT-PCR. It appears that the non-neuronal cholinergic system is involved in lung physiology and lung cancer. The physiological consequences of the presence of non-neuronal ACh will depend on the particular cholinergic signalling network of each cell type. Clarifying the pathophysiological actions of acetylcholine remains an essential task and warrants further investigation.

Key words: acetylcholinesterase; proliferation; airway surface fluid; lung; cancer.

INTRODUCTION

As a neurotransmitter, acetylcholine (ACh) plays pivotal roles in both the central and peripheral nervous systems. ACh is also a signalling molecule in the lung (for review see refs 1;2 and also below). Thus, ACh plays two different biological roles, acting as a classical neurotransmitter (neuronal) and as a cytomolecule (non-neuronal). The presence of cholinergic components in epithelial cells (intestine, skin, urogenital tract), mesothelial, endothelial, muscle and immune cell tissues points to the existence of a cholinergic phenotype, modulating basic cell functions such as gene expression, proliferation, differentiation, cellular adhesion, migration, secretion and absorption.

Multiple reports have established that human lung cells express the non-neuronal cholinergic system (reviewed in Ref. 3, i.e. the synthesizing enzyme, choline acetyltransferase (ChAT) [3-5], nicotinic (nAChR) and muscarinic (mAChR) receptors [6-11], as well as enzymes for ACh hydrolysis, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [12;13].

In the lung, the role of ACh as an autocrine/paracrine signalling molecule is not yet generally accepted despite many data showing that lung cells synthesize the cholinergic components [14]. Importantly, the case for non-neuronal autocrine signalling has also been reported in lung cancers [6;15;16]. The fact that nicotine regulates cellular proliferation by activation of *Akt* [17], or by β -arrestin-mediated activation of Src and Rb-Raf-1 pathways [18], at the same time as blocking apoptosis [16;19] provides strong evidence that stimulation of nicotinic and/or muscarinic receptors by endogenous acetylcholine or exogenous agonists associated to tobacco consumption could participate actively in lung cancer pathogenesis. The biochemical events between ligands and receptors leading to carcinogenesis and tumour progression are not fully understood. Another point of growing interest links the cholinergic system and angiogenic activity in tumours. Nicotine and endogenous cholinergic pathways could mediate on endothelial cell growth and angiogenesis [20-22].

Increasing evidence has shown that AChE may be involved in pivotal processes of carcinogenesis and tumour progression. Thus, in leukaemia, ovarian carcinoma and tumour cell lines, aberrations in the AChE gene are frequently found (reviews in [23-25]. A role of AChE expression during apoptosis has been demonstrated [26;27] and the mechanism underlying AChE upregulation is mediated by the JNK/c-Jun pathway [28]. Recently, we have shown that post-translational processing of AChE is altered in lung cancer in a cell type-dependent manner [13].

Our goal in the present study was to determine the level of acetylcholinesterase activity, to study the pattern of molecular forms and to detect the expression of cholinergic components important to cholinergic signalling. We show that acetylcholinesterase activity was found in bronchial aspirates and the differences between lung diseases have potential diagnostic uses.

MATERIAL AND METHODS

Chemicals

Acetylthiocholine iodide, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), tetraisopropyl-pyrophosphoramidate (*iso*-OMPA), polyoxyethylene₁₀-oleyl ether (Brij 96), enzyme markers for sedimentation analysis (bovine liver catalase, 11.4S, S_{20,w}, Svedberg Units, and bovine intestine alkaline phosphatase, 6.1 S_{20,w}), anti-goat IgG monoclonal antibodies conjugated with horseradish peroxidase and recombinant human AChE were purchased from Sigma Chemical Co. (Madrid, Spain). Goat antisera against the N-terminal peptide of human AChE (N19) was purchased from Santa Cruz Biotechnology Inc (CA, USA). Molecular weight protein standards were provided by Invitrogen. For RT-PCR assays, mRNA was extracted using a Chemagic mRNA Direct Kit (Chemagen AG, Germany). The GenAmp RNA PCR kit (Applied Biosystems, Branchburg, NJ, USA) was used for reverse transcription. Other chemicals were of analytical grade.

Patients

We studied 105 patients admitted to the *Virgen de la Arrixaca* University Hospital in Murcia (Spain) between 2003 and 2006, in all of whom pulmonary function was affected. Haemolytic bronchial aspirates (n=20) were discarded. A total of 85 samples of bronchioalveolar aspirates from patients without (control; n=49) or with lung cancer (n=36) were studied. Of them 70 were male and 15 female and their ages ranged from 20 to 88 years. The mean age was 62.60 years.

The histological evaluation of the tumours according to WHO criteria, revealed that 10 corresponded to adenocarcinoma (AC), 15 to squamous cell carcinoma (SCC) and 11 to small-cell lung carcinoma (SCLC). Staging was assessed according to the TNM classification system (Tumor Node Metastasis Staging System). All patients gave their consent after being appropriately informed.

Cell culture

The human NSCLC cell lines NCI-H1264, NCI-H157, A549, H23, and the human SCLC DMS-79, NCI-H69, NCI-H187 and N-417 were a gift of Dr. L. Montuenga, Department of Histology and Pathology, Schools of Sciences and Medicine, University of Navarra, and M. Sanchez-Cespedes, Lung Cancer Group, Pathology Programme, Spanish National Cancer Centre (CNIO). Caco-2 colonic cancer cell line was a gift of SACE (University of Murcia). The human breast cancer cell line MCF-7 and human melanoma SK-MEL-28 were purchased from the American Type Culture Collection. Cell lines were maintained in RPMI medium supplemented with fetal bovine serum (10% v/v), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 ug/ml) at 37°C in an atmosphere of 5% CO₂.

Processing of the samples

Bronchial aspirates were performed by the staff physicians in a standard fashion using a 8.0-mm fiberoptic bronchoscope. Subjects were given local anesthesia using 4% lidocaine sprayed in the oropharynx and 1% lidocaine instilled over vocal cords, carina and bronchus. The bronchial aspirates were centrifuged for 10 minutes at 1,500 rpm to pellet the cells. All fractions were rapidly frozen at -80°C until required. Serum from patients was obtained by drawing whole blood through standard procedures on the same day before the collection of bronchial aspirates.

Cytological examination of the cellular fraction of bronchial aspirates was performed by the pathologists and only cases with unequivocal malignant features allowing tumour typing were considered. Cellular fraction of bronchial aspirates was found to contain ciliated cells in much greater proportion than squamous cells and macrophages. Both, the number of recovered cells and the percentage of viable cells (as estimated by trypan-blue staining) varied significantly between samples. The number and percentage of cancerous cells in bronchial aspirates from lung cancer patients ranged from a few cells to several thousand.

Assay of the AChE activity in bronchial aspirates.

Recovered bronchial aspirates are variable mixtures of sterile saline solution, airway surface fluid (ASF) and ASF components. Therefore, the concentration of recovered molecules in the ASF is usually very low because of the saline solution

added. Measurement of AChE activity in such samples required a very sensitive methodology. The Ellman Method [29] is not sensitive enough to measure AChE activity in such diluted samples. AChE activity was determined using the Acetylcholine/Acetylcholinesterase Assay kit (A12217, Molecular Probes) according to the manufacturer's instructions. AChE activity bronchial aspirate fraction was measured by adding 0.1 ml of the serially diluted samples in duplicate onto 96-well microplates. After that, 0.1 ml of the mixture reaction containing 50 mM Tris-ClH, pH 7.5, 0.2M Amplex Red reagent, 2 U/ml horseradish peroxidase, 0.2 U/ml choline oxidase was added. Reactions were measured using a 530 nm excitation wavelength and a 590 nm emission wavelength, for 30 minutes at multiple time points to follow the kinetic. A standard curve ranging from 0 to 20 mU/mL of AChE from electric eel (included with the assay kit) was used in each experiment. One unit of AChE activity represents the hydrolysis of 1 μ mol of substrate per minute at 37°C.

Standardization of AChE activity in bronchial aspirates with Urea

It is difficult to estimate the actual concentration of recovered molecules in ASF *in situ* because when the saline solution is recovered by aspiration, ASF and its components are recovered along with it. The degree to which the process of collecting bronchial aspirates dilutes the airway surface fluid was determined using urea as a marker of dilution [30]. Serum and ASF urea concentrations were determined using the kinetic UV assay for urea (Roche Diagnostic) according to the manufacturer's instructions in a microplate spectrophotometer (Biotek Instrument Inc.) and using a standard curve ranging from 0 to 20 mg/dL urea. Concentration in ASF = (Concentration in samples x serum urea concentration) / (sample urea concentration).

Solubilization of AChE from lung epithelial and cultured cells

AChE was extracted from the airway cells by homogenization in 0.5 ml Tris-saline buffer (TSB; 1M NaCl, 50mM MgCl₂ and 10mM Tris, pH 7.5) supplemented with Brij 96 (0.25% w/v). After centrifugation at 30,000 rpm in a TLA Beckman rotor, 30 min at 4°C, the supernatant containing the soluble and membrane-bound AChE molecules was recovered and stored at -80°C until use. AChE activity in supernatants was measured by the Ellman method [29]. AChE was assayed with 1mM acetylthiocholine and 100 μ M *iso*-OMPA (an inhibitor of BChE). One unit (U) of AChE

activity represents the hydrolysis of 1 μmol of substrate per minute at 37°C. The hydrolysis of substrate due to unspecific esterases was negligible.

The amount of protein was determined by the Bradford method [31] with bovine serum albumin (BSA) as the standard.

Sedimentation analysis

The molecular distribution of AChE in both bronchial cells and fluids was studied by sedimentation analysis in sucrose continuous gradients as reported before [13]. Briefly, samples and sedimentation markers (bovine liver catalase and intestine alkaline phosphatase) were layered on the top of centrifuged tubes containing 5-20% sucrose gradients, in the presence of 0.5%w/v Brij 96 detergent. The gradient tubes were centrifuged at 35,000 r.p.m. in a SW41Ti Beckman rotor (Fullerton, CA, USA), 18h at 4°C. After centrifugation, fractions of 250 μl were collected from the bottom of the tube and assayed for AChE activity and enzyme markers to obtain the sedimentation profiles.

Reverse transcriptase -polymerase chain reaction

The expression of the components of the cholinergic system, such as the enzyme that synthesizes ACh, choline acetyl-transferase (ChAT), the enzyme that hydrolyzes it (AChE) and nicotinic receptor subunits (nAChR) was studied by RT-PCR. For this purpose, mRNA was extracted from cultured human tumour cells using the Chemagic mRNA Direct Kit (Chemagen), and reversed transcribed into cDNA by random priming (GeneAmp RNA PCR Kit, Applied Biosystems). Subsequently, 2-5 μL of cDNA was amplified by 40 cycles of PCR (10 seconds to 95°C, 10 seconds at 55-60°C, 15 seconds at 72°C) at a LightCycler Instrument (Roche) using the primer pairs listed in Table 1. Negative controls (without reverse transcriptase) for each primer pair were assayed. The primer pairs employed to detect the mRNA of the cholinergic components are listed in Table I, where the strategy (intron-spanning/intron-flanking) employed is indicated. β -actin-mRNA was used as an internal control. The PCR products were visualized by electrophoresis on 1.5% agarose gels.

Western blotting

AChE subunits from both ASF and bronchial cells were resolved by reductive SDS-polyacrylamide gel electrophoresis [32], carried out in 12.5% polyacrylamide-gel

slabs. Proteins were electro-transferred to PVDF membranes, blocked with 5% non-fat dried milk and incubated with the N19 anti-AChE antisera (Santa Cruz). Since N19 antibodies are produced against the N-terminal peptide of human AChE, it should react with the full set of AChE variants. Negative control samples (without primary antibody N-19) lacked of staining bands and verified that protein bands correspond with proteins specifically recognized by the primary antibody. Labelled proteins were revealed with anti-goat IgG horseradish peroxidase -conjugated antibodies and the ECL Western detection system (GE Healthcare UK). Blots were exposed to Hyperfilm-ECL (Amersham Biosciences UK Ltd) for various periods of time. The size of the AChE subunits was estimated in reference to appropriate protein standards (ECL DualVue markers from GE Healthcare), and the intensity of the protein bands was quantified using GelPro Analyzer Software (version 3.1; Media Cybernetic). β -actin was used as a loading control and positive control was performed loading 1, 5 or 10 μ g of recombinant AChE (Figure 2).

Statistical analysis

The results are given as a mean \pm SEM. The statistical significance of the difference in ACh content and AChE activity between cancerous and non-cancerous groups was evaluated by Student's *t*-test. The non-parametric Mann-Witney test (signed rank test at $P < 0.05$) was used to compare AChE between the several histological types of lung cancer. Statistical analysis was performed using the SPSS software program (version 10 for Windows; SPSS, Chicago, IL).

RESULTS

AChE activity in ASF from control and cancerous patients

Airway surface fluid contained AChE activity. The AChE activity in non-cancerous samples was 1.34 ± 0.17 mU/mL and decreased to 1.18 ± 0.18 mU/mL in tumoral bronchial aspirates (Table II). Bronchial aspirates from patients diagnosed with SCLC, AC and SCC showed statistically significant differences in AChE activity (Table II). Bronchial aspirates from AC patients expressed 2.17 ± 0.44 mU/mL AChE activity ($n=7$), SCLC samples expressed 0.91 ± 0.24 μ U/mL ($n=10$) and SCC samples dropped to about 0.51 ± 0.24 μ U/mL ($n=10$). We have previously reported that AChE activity varied according to the histological classification of lung tumours [12]. In accordance with these previous data, AChE activity in ASF from SCC dropped greatly. No

significant differences were observed in the AChE activity of ASF from patients with a smoking habit, COPD, bronchitis or pneumonia.

AChE molecules in bronchial aspirates

The pattern of molecular forms of AChE in both soluble and cellular fractions of bronchial aspirates was investigated by sedimentation analysis (Figure 1). Abundant 4.4 ± 0.2 S (Svedberg units) and less 5.9 ± 0.2 S and 2.8 ± 0.1 S AChE forms were identified (Figure 1). These molecular forms corresponded to dimeric (G_2^A ; 4.5 S) and monomeric (G_1^A ; 2.8 S) amphiphilic AChE molecules, while 5.9 S molecules were assigned to hydrophilic dimers of AChE (G_2^H). All the samples included in the study gave a similar pattern of AChE forms, indicating that whatever the pulmonary diseases are they do not affect the biosynthesis and/or secretion of AChE molecules. Sedimentation analysis of cellular extracts from cultured cancer cells of epithelial origin such as lung (A549, H157, N417 and H69), breast (MCF-7) or colon (Caco-2) showed almost identical AChE molecular composition. Tetrameric globular forms (G_4) were only detected in small cell lung cancer cells and their contribution was always scarce (Figure 1B).

Detection of AChE proteins in bronchial aspirates by western blotting

We have previously reported that lung cancer tissues express AChE molecules capable of hydrolysing ACh and also molecules with the same apparent molecular weight but that are not catalytically active [11]. To gain an insight into the differences in the size as well as the content of inactive AChE molecules, we decided to assay an immunoblot of both soluble and cellular fractions of bronchial aspirates. For this, samples were subjected to reductive SDS-PAGE, transferred to PVDF membranes and then incubated with antibodies able to detect all AChE molecules. Abundant 50 kDa and less 70 kDa subunits were recognized (Figure 2). As in lung tissues [13], no correlation was found between the AChE units loaded and the labelling intensity of bands demonstrating the presence of inactive AChE molecules in ASF. The potential use of the following ratio: "AChE units/intensity of labelling" (i. e., the relative content of inactive AChE) as a diagnostic tool is under investigation.

mRNA expression of cholinergic components in the lung.

The role of the autocrine growth factor in lung cancer growth has been well-established [6]. Analysis of human lung cancer cell lines by real time PCR shows the expression of mRNA of the enzyme responsible for (a) the synthesis of ACh (CHAT); (b) nicotinic receptor subunits (nAChR); and (c) hydrolysing enzymes (AChE and BChE) (Figure 3). All cell lines analysed expressed ChAT, AChE and BChE needed for the synthesis and degradation of ACh. The Beta4 nicotinic subunit was present in all lung cell lines, except in H157, H187, N417. SK-MEL-28 but not MCF7 expressed beta4. Meanwhile, alpha7 and alpha3 nicotinic subunit mRNA was detected in all cell lines. The expression of mRNA coding for the cholinergic components did not indicate any specific pattern either associated with the tissue origin or to the histology of the tumours from which the cell lines are derived.

DISCUSSION

This study presents data showing that human lung epithelium expresses the components for establishing a physiologically active cholinergic system, possibly needed for a correct pulmonary function and involved in cancer growth. This study shows that: (1) ASF contains enough AChE activity to control the effective levels of the neurotransmitter and/or its degradation product choline; (2) AChE activity varies according to the histological type of lung tumours being the highest in ASF from lung adenocarcinomas and the lowest in squamous cell lung carcinomas; (3) The pattern of molecular forms of AChE do not change with cancer; (4) As in lung tissues, inactive AChE molecules are present in bronchial aspirates, increasing their content in cancerous samples and showing histological-type differences; and (5) both small- and non-small-cell lung cancer cells express genes needed for a cholinergic autocrine or paracrine loop.

It has been clearly demonstrated that nicotine and other tobacco components increased the proliferation of non-cancerous and cancerous [6;18;33;34] human lung cell lines, at the same time as acetylcholine or carbachol also modulated the proliferation of lung cells [6;35]. By using agonist or antagonist of nicotinic and muscarinic receptors the growth of lung cells, and other cell lines, is affected (for review see refs [15;36;37]. It becomes evident that cholinergic signalling can modulate cellular phenotype and function. Further, it appears likely that nicotine plays its pathological role through its direct interaction with cholinergic receptors [18;20;37]. The recent studies elucidating the events implicated in these receptor-dependent

responses [6;17;36-39] are important not only for tobacco consumption-associated cancer but also for lung tumours, such as adenocarcinomas that have a weaker correlation with smoking than SCC and SCLC.

According to RT-PCR, our results are consistent with others (revised in refs 1, 2) showing that lung cells express mRNAs needed for establishing functional cholinergic systems in both small-cell and non-small lung cancer cells. Nevertheless, as far as we know there is not an association between particular cholinergic gene expression patterns and lung cancer pathogenesis. A very interesting study presented by Minna et al. [40] shows that tobacco consumption could change the composition of nicotinic receptors of bronchial epithelial cells. It remains to be seen if these changes are important for the pathogenesis of lung cancers but they clearly indicate the existence of particular nAChR subunit gene expression patterns which change upon several physiological conditions. As ACh levels could also change with other pathologies, for example in response to inflammation processes [1;41], the same pathways that are involved in nicotine-associated lung cancer progression could also play a role in the biology of lung cancer not linked to tobacco consumption.

Our results show that airway surface fluid contains an important level of AChE activity which varies depending on the histology of the lung carcinoma (Table II). AChE activity in ASF was higher than the control in AC, and lower in SCLC and SCC. We have previously shown that surgery pieces from SCC express very less AChE activity than non-cancerous and AC samples [13]. It is interesting that ASF from smoking-associated lung cancers such as SCC and SCLC contain less AChE activity than ASF from controls or AC. The actual role of AChE activity in cancer is controversial. Cancer promotes changes affecting both the AChE gene structure and expression [42;43] and the protein properties [13;44-47]. More recently, it has been proposed that AChE plays a role in apoptosis [26-28] while immunohistochemistry staining is inversely associated with survival in ovary cancer [48].

AChE biology is a very complex process and the precise physiological role of this enzyme in non-neuronal contexts is poorly understood. AChE can be found as soluble or as membrane-bound subunits and little is known about the regulation and its physiological significance. Whether secretion of AChE molecules to epithelial lining fluid of the bronchi is a controlled process needed for the normal pulmonary function need to be further investigated. Thus, it cannot be assumed, but also it cannot be excluded, a correlation between the AChE activity in the fluid of aspirates and in the

malignant epithelium cells. A correlation between AChE activity in both airway surface fluid and surgical pieces in squamous cell carcinomas (SCC) with a great reduction of the AChE activity was found, whereas no correlation between AChE activity in ASF and surgical pieces derived from adenocarcinoma (AC) was observed. We discussed in our previous work [13] that the heterogeneous cellular origin of lung cancers included in the AC group may mask possible differences, and the same can happen with the results of AChE activity in the airway surface fluid. It is tempting to think that in AC the secretion of active AChE is up-regulated and that this phenomenon affects the tumour biology. By other hand, the differences between the AChE activity in airway surface fluid from AC and SCLC could be of diagnostic interest.

With regard to the molecular composition of AChE, sedimentation analysis revealed that ASF contains abundant amphiphilic dimers (G_2^A) and fewer monomers (G_1^A) (Figure 1), the same pattern as in the cellular fraction of bronchial aspirates. The similarity between the ASF AChE pattern and the expression by tumours of epithelial origin such as lung [13], breast and metastatic lymph nodes [44;45] and colon [49] reinforces the belief that human epithelia only express light molecules of AChE as enzymatic active forms. As human serum contains a very small amount of AChE activity and great butyrylcholinesterase activity, and the opposite is found in ASF, it is also probable that ASF AChE comes from the bronchial epithelium. The pattern of AChE molecular forms from cultured cancer lung cells also shows that only small-cell lung cancer cells express AChE tetramers and this seems to be related to adhesion-independent growth but not to the histological type.

Immunoblotting results indicate that the same labelled protein bands are found in ASF (Figure 2) and cellular fraction of bronchial aspirates (not shown). It is remarkable that ASF contains inactive AChE molecules as occurs in lung tissue (50 kDa protein band) and active AChE subunits (about 70 kDa). In agreement with previous work [13], squamous cell carcinoma showed the lowest content of active subunits (lane S, Figure 2). We have previously assigned the 50 kDa protein band to the stress-induced AChE variant (AChE-R), which is synthesized more abundantly in lung cancer tissues and especially in SCC. Interestingly, Soreq's Group found that CREB's basal levels were insufficient to block the AChE-R proliferative effect in AChE-R transfected glial cells and hypothesized that this could increase the risk of tumour growth in individuals exposed to anti-cholinergic agents [50]. The potential diagnostic use derived from this

study and the physiological significance of cholinesterases in lung cancer warrants further investigation.

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Conflict of interest statement

None declared

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Legend to Figures

Figure 1. Sedimentation profiles with AChE forms in bronchial aspirates and cultured lung cells. *A*, AChE forms in the soluble (ASF) and the cellular (Cells) fraction of bronchial aspirates were identified by sedimentation analysis in sucrose gradients (5-20%) containing Brij-96. Sedimentation coefficients were calculated by taking internal sedimentation markers (C, catalase 11.4S; P, alkaline phosphatase 6.1S) as the reference. Figure shows a profile corresponding to a cancerous ASF and cellular fraction. No differences between non-cancerous and cancerous samples were observed. *B*, Enzymatic extracts from NSCLC (A549, H157), SCLC (N417, H69), breast cancer cells (MCF7) and colonic cancer cells (Caco-2) were fractionated by sucrose gradients centrifugation as before. Amphiphilic globular AChE dimers and monomers appear in all samples but globular tetramers are restricted to cells that grew unattached to the culture plate. AChE activity is expressed in arbitrary units.

Figure 2. Immunoblotting of AChE protein in airway surface fluid. Proteins (50 µg) in non-tumoral (NT), adenocarcinoma (A), squamous cell carcinoma (S) and small-cell lung carcinoma (SCLC) ASF were resolved by reductive SDS-PAGE as described in material and methods. N19 antibodies recognize the complete set of AChE variants. According to previous data [13], the labelled bands were assigned to the synaptic AChE (variant T; 68 kDa) and to the read-through AChE-R (stress-associated; 50 kDa). Note the weaker labelling of the catalitically active AChE subunit (68 kDa) in squamous cell cancer (S) and the stronger labelling of the 50 kDa protein band in tumoral ASF. Right panel shows the correlation between AChE activity loaded and intensity of labelling.

Figure 3. Expression of cholinergic components in cultured cancer cells. mRNA was prepared and RT-PCR performed for the given cell lines with primers shown in Table 1. "AChE T"= acetylcholinesterase variant T. "AChE H"= acetylcholinesterase variant H. "AChE R"= acetylcholinesterase variant R. "E1d"= N-terminus extended acetylcholinesterase. "BChE" = butyrylcholinesterase. "ChAT" = choline-acetyltransferase. "Alpha3" = nicotinic acetylcholine receptor α 3 subunit. "alpha7" = nicotinic acetylcholine receptor α 7 subunit. "Beta4" = nicotinic acetylcholine receptor β 4 subunit. No products were visualized when RT-PCR was performed in the absence of reverse transcriptase (non shown)

Table 1. RT-PCR primers		
Gene	Forward Reverse	Lenght (bp)
AChE variant T	5' AACTTTGCCCGCACAGGGGA 5' GCCTCGTCGAGCGTGTCCGT	203 (1)
AChE variant H	5' AACTTTGCCCGCACAGGGGA 5' GGGAGCCTCCGAGGCGGT	201 (1)
AChE variant R	5' CCCCTGGACCCCTCTCGAAAC 5' ACCTGGCGGGCTCCCACTC	315 (2)
AChE E1d	5' CCTGGTGACGAAAGTCCGA 5' TCCTCCACCCAGGAGCCAGAG	247 (2)
BChE	5' TGTCTTTGGTTTACCTCTGGAA 5' CACTCCCATTCTGCTTCATC	297 (2)
Chat	5' TAGCCAGGTGCCCACAAC 5' ATGAGGCTTTCTTCCACAGC	177 (1)
Alfa 7	5' CAGTACTTCGCCAGCACCAT 5' AGCCGATGTACAGCAGGTTC	283 (2)
Alfa 3	5' GAAGCCAAAGAGATTCAAGATG 5' TTGCAGAAACAATCCTGCTG	114 (1)
Beta 4	5' GACCAGAGTGTTCGTTGAGGA 5' GGTAGGAAGAGCCCCACAGT	110 (1)
β-actin	5' AGAAAATCTGGCACCACACC 5' GGGGTGTTGAAGGTCTCAA	143 (2)

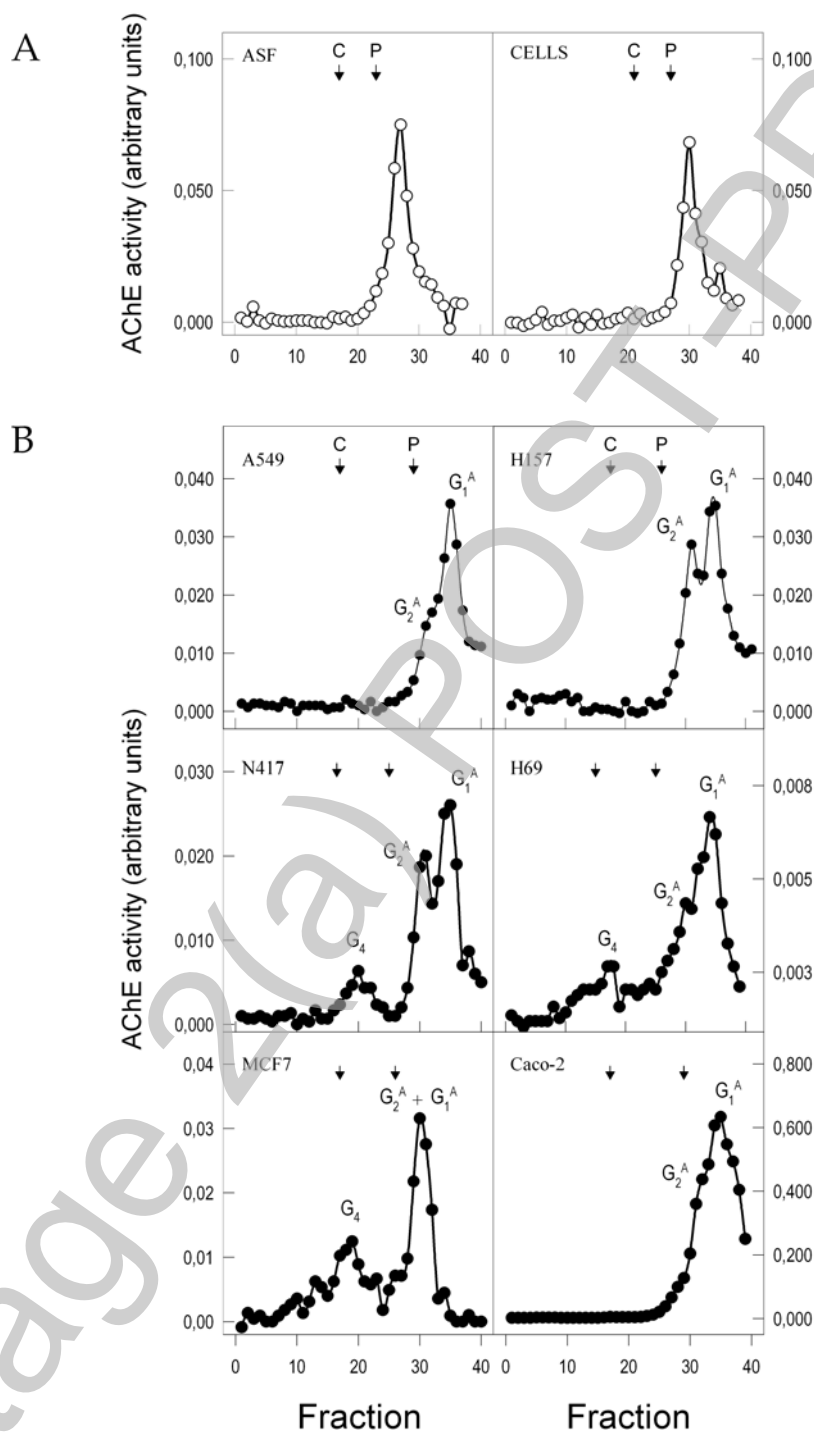
(1) Intron-spanning, (2) Intron-flanking

Table II. AChE activity in airway surface fluid of bronchial aspirates

Diagnosis	AChE [mU/mL] Mean \pm SEM (n)	Median, I-III quartile	<i>P</i> -value Student's <i>t</i> -test
Non-Cancerous	1.34 \pm 0.17 (37)	0.542, 1.111-2.082	0.434
Cancerous	1.18 \pm 0.18 (35)	0.249, 0.853-1.807	
AC	2.17 \pm 0.44 (7)	1.270, 2.626-3.013	
SCC	0.51 \pm 0.23 (10)	0.274, 0.225-0.662	
SCLC	0.91 \pm 0.24 (7)	0.270, 0.876-1.485	
COPD	0.93 \pm 0.47 (6)	0.201, 0.529-1.613	
Bronchitis	0.56 \pm 0.13 (9)	0.225, 0.392-0.898	
Pneumonia	0.66 \pm 0.27 (4)	0.191, 0.558-1.222	
Smoking	0.92 \pm 0.18 (25)	0.175, 0.629-1.490	
			<i>P</i> -value
			Mann-Witney <i>U</i> -test
Non-cancerous vs AC			0.038*
Non-cancerous vs SCC			0.007*
Non-cancerous vs SCLC			0.431
AC vs SCC			0.012*
AC vs SCLC			0.053
SCC vs SCLC			0.133
COPD vs non-COPD			0.616
Bronchitis vs non-Bronchitis			0.143
Pneumonia vs non-Pneumonia			0.607
Smoking vs non-Smoking			0.924

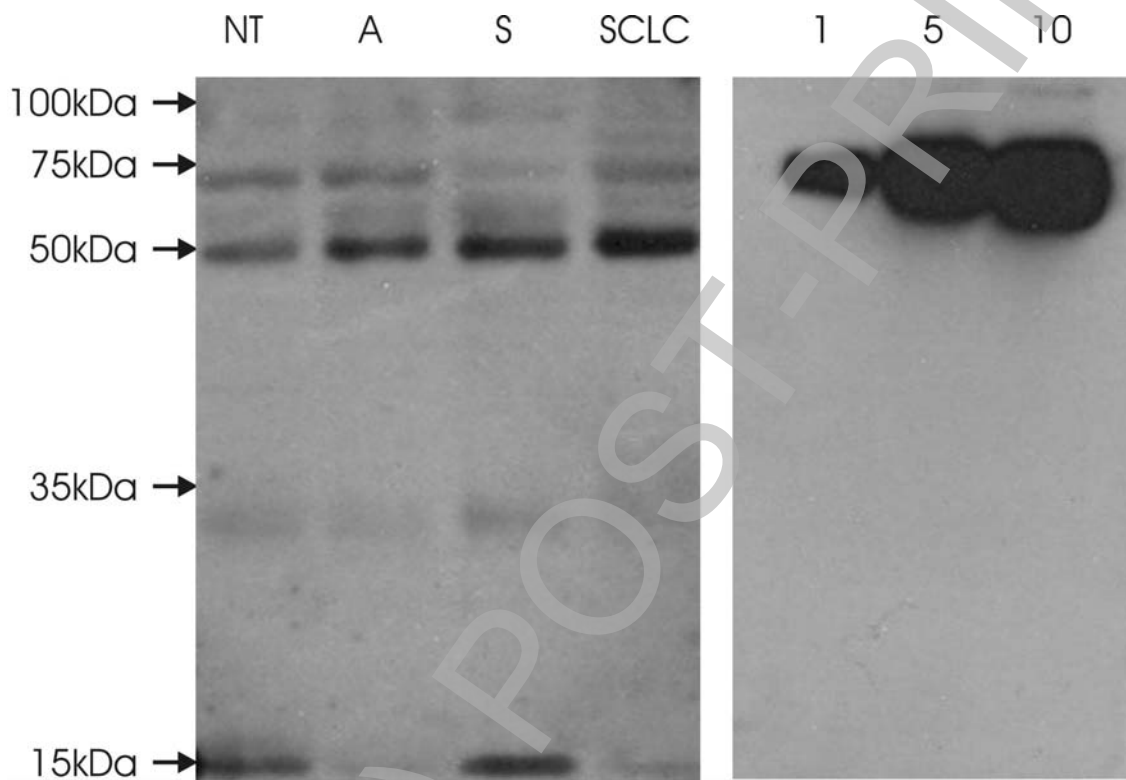
AChE activity is given as mean \pm SEM, with the number of samples included in brackets. Median, I and III quartile are also shown. Statistical difference between AChE activity in non-cancerous and cancerous samples was evaluated by Student's *t*-test. The non-parametric Mann-Witney *U*-test was used to determine statistical significance ($p < 0.05$) according to histology of lung cancer. AC = Adenocarcinoma; SCC = Squamous cell carcinoma; SCLC = Small-cell lung cancer; COPD = Chronic obstructive pulmonary disease. * Statistically Significant Differences

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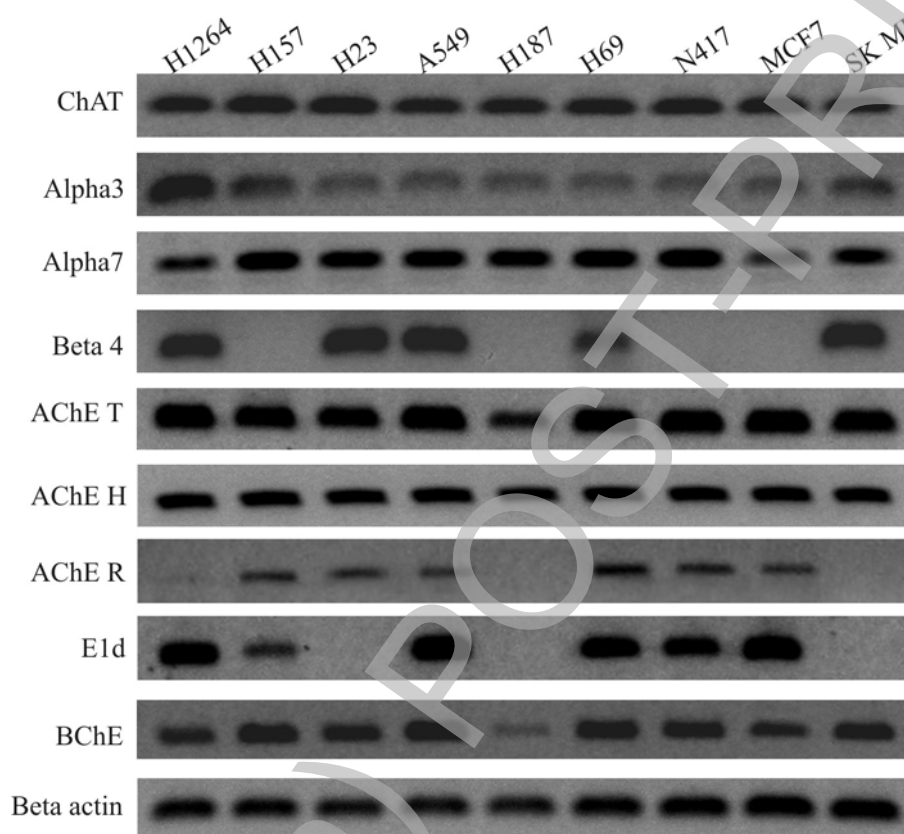
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