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Activated cholinergic signaling provides a target in squamous cell lung carcinoma

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Abstract

The binding of exogenous nicotine to nicotinic acetylcholine receptors (nAChR) and the binding of endogenous acetylcholine to both nAChR and muscarinic acetylcholine receptors (mAChR) stimulates growth of both small cell and non-small cell lung carcinomas. Understanding how cholinergic signaling is upregulated in lung cancer may suggest new therapeutic approaches. Analysis of 28 squamous cell lung carcinomas (SCC) showed increased levels of *a*5 and β3 nAChR mRNA and increased levels of acetylcholine associated with increased levels of ChAT mRNA and decreased cholinesterase mRNAs. Lynx1, an allosteric inhibitor of nAChR activity, was also decreased in SCC. Thus cholinergic signaling is broadly increased in SCC caused by increased levels of receptors, increased levels of ligands and decreased levels of receptor inhibitors. Partially explaining the cholinergic upregulation seen in SCC, incubation of the H520 SCC cell line with nicotine increased levels of ACh secretion, increased expression of nAChR and, as measured by electrophysiologic recording, increased activity of the expressed nAChR. Consistent with these effects, nicotine stimulated proliferation of H520 cells. One approach to blocking proliferative effects of nicotine and acetylcholine on growth of lung cancers may be through M3 mAChR antagonists which can limit the activation of MAPK that is caused by both nicotinic and muscarinic signaling. This was tested with the M3-selective muscarinic antagonist darifenacin. Darifenacin blocked nicotine-stimulated H520 growth *in vitro* and also blocked H520 growth in nude mice *in vivo*. Thus cholinergic signaling is broadly upregulated in SCC and blocking cholinergic signaling can limit basal and nicotine-stimulated growth of SCC.

Keywords

Lung cancer; nicotine; nicotinic receptors; muscarinic receptors; acetylcholine

Introduction

Lung cancer is the number one cause of cancer death in the United States with deaths in 2007 estimated to exceed 160,000 (1). Lung cancer is classified into small cell lung carcinoma

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(SCLC) which accounts for approximately 14–20 percent of cases and non-small cell lung carcinoma (NSCLC) which accounts for the remaining cases. NSCLC consist primarily of squamous cell carcinoma (SCC) and adenocarcinoma. Despite improvements in responses to increasingly sophisticated combination of surgery, radiation and chemotherapy (2), survival remains low. Thus the development of new therapeutic approaches is clearly needed.

Smoking is associated with the vast majority of lung cancer (3). While the primary mechanism of smoking-induced carcinogenesis derives from carcinogens in smoke, recent data shows that nicotine and nitrosamines in smoke bind to nicotinic acetylcholine (ACh) receptors (nAChR) on lung cancers to stimulate growth and potentially tumor progression (4–9). Expression of nAChR is seen in both SCLC and NSCLC (4,10–12). Expression of nAChR in lung cancers derives from expression of nAChR in normal lung cells. Normal bronchial epithelial cells express nAChR as part of a cholinergic autocrine loop (13–16) in which all proteins needed for cholinergic signaling are present including the ACh-synthesizing enzyme choline acetyltransferase (ChAT), the vesicular acetylcholine ACh transporter (VAChT), the high-affinity choline transporter CHT1, the ACh hydrolyzing enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), nicotinic acetylcholine receptors and muscarinic acetylcholine receptors (mAChR). We and others have also demonstrated that the interaction of ACh with both nAChR and mAChR stimulates cell proliferation (11,17–20) and we have previously reported that SCLC similarly express this cholinergic autocrine loop and that muscarinic antagonists can inhibit SCLC cell proliferation.

While the expression of nAChR in SCC has been described, the expression of the complete cholinergic autocrine loop in SCC has yet to be described. In this paper we show that SCC like SCLC express a cholinergic autocrine loop. Chi-leung Lam et al (10) recently reported in a series of NSCLC (predominantly adenocarcinomas) that some nAChR are changed in tumor relative to normal lung and that nicotine exposure appears to change receptor expression. As we demonstrate here, changes in cholinergic signaling in SCC are not limited just to nAChR expression but large changes in ACh synthesis and degradation are also present. Given the upregulation of cholinergic signaling in lung cancer, the role of smoking and continued stimulation of cancer cells by nicotine is of obvious importance. The interaction of nicotine with its receptor can lead to receptor inactivation or receptor activation depending on receptor subtype and cell type. Here we show that nicotine upregulates both nAChR expression and activity.

Dennis and co-workers have demonstrated Akt activation by nicotine (4,21), and our lab and other laboratories have also demonstrated MAPK activation by nicotine (6,22–24). Endogenous ACh released from lung cancers signals through both nAChR and mAChR and also leads to Akt and MAPK activation (22). Because MAPK is activated by both nicotinic and muscarinic pathways and we have previously shown that M3 muscarinic antagonists decrease basal MAPK activation as well as blocking ACh-induced MAPK and Akt, M3 muscarinic antagonists present a potential approach to blocking both nicotinic and muscarinic proliferative pathways. In this paper we also show that muscarinic M3 antagonists can block basal proliferation of SCC as well as nicotine-stimulated SCC proliferation.

Materials and Methods

SCC Cell lines and SCC samples

The SCC cell line H520 (ATCC, Rockville, MD) was used for most studies and maintained as recommended by ATCC. For analysis of expression of cholinergic RNAs and western blot analysis, 28 frozen archival SCC samples were obtained from the tumor bank of the Department of Pathology of the Oregon Health and Science University (OHSU). For 6 of the 28 samples, adjacent normal tissue was also available. H&E stained-slides of the samples were reviewed

to confirm diagnosis and establish degree of differentiation. For immunohistochemical analysis of cholinergic gene expression, another 31 archival, paraffin-embedded SCC samples were obtained from the of the OHSU Department of Pathology. For HPLC analysis of ACh content of SCC, an additional 8 SCC and 8 normal lung samples were obtained form the OHSU tumor bank. An additional 6 SCC cell lines (H647, H1703, H1869 H2066, HTB-58, HTB-59) from ATCC were also screened for cholinergic components and responses.

Real-time PCR and western blots

Real-time PCR was used to quantify cholinergic gene expression in tumor samples as previously described (11,25). Total RNA and protein in SCC and the normal lung tissues were prepared with TRI-reagent (Molecular Research Center Inc., Cincinnati, OH) (26). The probes and primers used in real-time PCR reactions were as described previously (11) or are listed in supplement table 1. All reactions were run in triplicate and RNA levels were normalized to 18S RNA expression as an internal standard. In addition, no-reverse transcriptase controls were run with all RNAs to check for genomic DNA contamination. Western blot analysis was performed as described previously (25) using actin as an internal standard. Antibodies are as described below for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed as previously described (11,15,22). Antibodies used were anti-choline acetyltransferase (MAB305); anti-AChE (MAB337) from Chemicon International, Inc (Temecula, CA); anti-VAChT (H-V005) from Phoenix Pharmaceuticals (Belmont, CA); anti CHT1 generously provided by R. Blakely (11) and anti-lynx1 as previously described (25). Muscarinic receptor antibodies were as follows: anti-M2 (WR-3791 from R&D Systems (Minneapolis, MN) (SC-9346) and anti-M3 (H210) from Santa Cruz Biotechnology; (11,15,25). Nicotinic receptor antibodies were as follows: anti-α3 (mAb356), anti-α4 (mAb299), anti-α7 (mAb319), anti-β2 (mAb3724), anti-β4 (mAb337) from Lindstrom and coworkers (11,15) and anti-β3 (SC-9346) from Santa Cruz Biotechnology. Immunostaining was independently scored by 2 readers on a scale from 0 – 4 where 0 = no staining, 1 = focal weak staining, 2 = diffuse weak staining or focal strong staining, 3 = diffuse medium staining, 4 = diffuse strong staining. The readers were blinded to each others results and other reads on the slides.

ACh Assay

ACh synthesis by SCC was assayed by HPLC as described previously (11). For measurement of ACh in tissue, 100–200 mg samples were homogenized in 2ml of 90 % methanol containing 0.1 M formic acid, and then centrifuged at 10,000 rpm for 15 min. One ml supernatant was lyophilized and stored at −80 °C. The dried samples were dissolved in 80–120 μl of the HPLC mobile phase, filtered and 10 μl injected into the HPLC. Each sample was measured at least in duplicate. 2nM standard ACh was added to 2 ml 90% methanol and processed as above for determination of ACh recovery. For measurement of ACh secretion by SCC cell lines, 500,000 cells in 1 ml fresh media were plated into each well of 24-well dishes and neostigmine (5×10^{-5} M, Sigma-Aldrich, St. Louis MO) was added to all dishes to inhibit ACh degradation. Twenty-four and forty-eight hours after plating, supernatants were collected, frozen on dry ice, and stored at − 80 °C.

Calcium fluorometry

Changes in SCC cell line intracellular calcium concentration $[Ca^{2+}]_i$ elicited by ACh was measured fluorometrically using a FLEXstation (Molecular Devices, Sunnyvale, CA, USA) as described previously (22). Drugs used (ACh, carbachol, mecamylamine and atropine; Sigma-Aldrich) were diluted to desired concentrations with Hank's buffered salt solution

supplemented by 20 mM HEPES plus 2 mM $CaCl_2$ and applied at the concentrations shown in the results.

Electrophysiology

Cells were transferred to a recording chamber mounted on the stage of a Leica microscope and perfused with Krebs solution with the following composition (in mM): 140 NaCl, 3 KCl, 1.8 CaCl2, 1 MgCl2, 10 HEPES, and 5 glucose, pH 7.3, osmolarity 300 mOsm. Cells were perfused at of 2–3 ml/min with bathing solution at room temperature. Drugs were applied by fast step perfusion system (SF-77B perfusion, Warner instruments, Hamden CT). To isolate inward currents, an internal pipette solution with following composition was used (in mM): 130 CsCl, 1 CaCl2, 2 MgCl2, 10 EGTA, 10 HEPES, and 4 Mg-ATP, pH adjusted to 7.2 with CsOH. Whole-cell currents were recorded from H520 cell using standard patch-clamp techniques (27). The resistance of the recording pipettes was 3–5 MΩ. Cells were voltage clamped at −60 mV. A Multiclamp 700B (Axon Instruments, Foster, CA) was used to record whole cell currents. Voltage clamp protocols, data acquisition, and analysis were performed using DigiData 1322A interface and pClamp9 software (Axon Instruments).

Cell proliferation Assay

H520 SCC cells were used to determine the ability of the M3 antagonist darifenacin to inhibit nicotine-stimulated proliferation. Cells were plated at a concentration of 2500 cells per well as described above. The following day cells were changed to media containing 1% fetal calf serum and drugs as indicated in figure 5. Cell density was monitored using the Cell Titer-Blue assay (Promega Corp, Madison, WI) as previous described (22).

Nude mice studies

For tumor xenograft growth studies, male mice (NU/NU, Charles River Laboratories) were injected with H520 SCC tumor cells, treated with the M3 receptor antagonist darifenacin and tumor growth monitored. Darifenacin was generously provided by Novartis Pharma AG (Basel, Switzerland). H520 cells were grown as described above and 5×10^6 cells wereinjected subcutaneously into the right flank of each mouse as described previously (22). Tumors were allowed to grow for one week, then drug administration initiated and continued for the next 4 weeks. Darifenacin was dissolved in 50% DMSO/50% phosphate-buffered saline and administered by subcutaneously-implanted osmotic minipumps (Alzet model# 2004) at doses of 6.0 mg/kg per day. Control animals received minipumps filled with 50% DMSO/50% phosphate-buffered saline. Tumor volume was determined weekly by measuring with calipers (volume = height × width × depth). The study was terminated after 4 weeks of drug administration. 10 animals were used per group. At sacrifice tumors were removed and weighed.

Statistical analysis

Data are presented as mean ± SE. Data of real-time PCR and western blot analysis were analyzed by T-test using NCSS 2004 statistical software (Kaysville, UT). EC_{50} and IC_{50} in the Calcium fluorometry were calculated with the SOFTmax program 4.0.1 provided by Molecular Devices Corporation (Sunnyvale, CA, USA). Cell proliferation was analyzed by 1 or 2 way ANOVA followed by Tukey-Kramer multiple comparison tests.

Results

Expression of cholinergic mRNAs and proteins in SCC

Real-time PCR was performed to characterize cholinergic gene expression in 28 SCC tumors and 6 adjacent normal tissues as described in the methods. Frozen SCC samples were obtained

from the OHSU tumor bank and diagnosis and histologic grade confirmed by examination of H&E stained-sections of the same tumors. As shown in figure 1A, the enzymes necessary for the synthesis and degradation of ACh were all present in SCC. ChAT was significantly increased in SCC as compared to adjacent normal tissue while levels of CHT1 and VAChT were not significantly changed (Fig. 1A). Levels of mRNA that encode proteins that serve to limit cholinergic signaling, AChE, BChE and lynx1 were all significantly decreased in tumors compared to controls (Fig. 1A). All nicotinic receptors measured in tumors trended higher than in normal tissues and levels of α5 and β3 were significantly increased in the tumors (Fig. 1B). Levels of M2 mAChR mRNA were significantly decreased in tumors compared to controls while significant differences between tumors and controls were not seen in levels of M3 and M4 mAChR mRNA (Fig. 1C). Changes in cholinergic protein levels as measured by Western blot analysis mirrored changes in RNA levels and significant decreases in levels of AChE, M2 and lynx1 between tumors and controls were observed (data not shown). Levels of M3 and M4 by western blotting were not significantly different between groups (data not shown). The differences in cholinergic mRNAs between tumors and normals tended to increase as tumors became less differentiated. Levels of ChAT, α5 nAChR and M3 mAChR trended higher in less differentiated SCC and levels of lynx1 and AChE RNA trended lower in less differentiated SCC (Fig. 1D).

Next the expression of cholinergic proteins in SCC by immunohistochemistry was determined in 31 SCC tumors (Fig. 2). As can be seen in figure 2A, consistent with the analysis of mRNA expression, the majority of SCC expressed the proteins needed to both synthesize ACh and to respond to ACh though both the nicotinic and muscarinic cholinergic pathways. Representative staining for ChAT, CHT1 and AChE is shown in figure 2B. Approximately 60% of SCC expressed ChAT by immunohistochemistry and essentially all expressed at least one subtype of nicotinic or muscarinic receptors. Thus the majority of SCC can synthesize ACh and almost all would respond to exogenous ACh or nicotine.

In addition, as listed in the methods, 7 SCC cell lines were analyzed for ChAT and cholinergic receptor expression. Similar to the tumor samples, all SCC cell lines examined expressed at least one subtype of nicotinic or muscarinic receptor and 6 of 7 cell lines expressed ChAT (data not shown). Six of seven SCC lines examined also showed increased intracellular calcium in response to ACh challenge. Because the H520 SCC cell line proved the most amenable for patch clamp analysis it was used for more detailed studies as described below.

ACh is elevated in SCC

Because the presence of the enzyme to synthesize ACh does not guarantee actual production of ACh, levels of ACh were assayed in 8 additional tumor samples and 8 normal lung samples (Fig. 3). As shown in figure 3A, high levels of ACh were present in 6 of 8 tumor samples but nearly undetectable in all but one normal lung sample. This is consistent with the dramatic difference in ChAT mRNA levels between tumor and adjacent normal tissues shown in figure 1A and the decreased levels of cholinesterase shown in figure 1D. As shown in figure 3B, nicotine exposure may play a role in upregulating ACh levels in tumors as incubation of the H520 SCC cell line with nicotine mildly, but significantly, increased ACh secretion by SCC cells under conditions (10% fetal calf serum) in which nicotine did not increase cell number..

Nicotine upregulates and activates nicotinic receptors in SCC

To assess how chronic nicotine exposure effects nAChR expression and activity, H520 cells were incubated with nicotine and activity of nicotinic receptors in SCC H520 cells was measured by whole cell voltage clamp. As shown in figure 4A, incubation in 2 μM nicotine for 48h upregulated nAChR expression as shown by immunostaining. As shown in figure 4B, the nAChR expressed in SCC are active with both α7 nAChR and non-α7 containing nAChR

currents. Application of 100 μM nicotine to a H520 cell voltage clamped at −60 mV elicited a transient inward current (Fig. 4B), and mean peak nicotine-induced inward current (I*nic*) was −120.4 ± 11.5 pA (n=9). Desensitization time constant for I*nic* was 8.9 ± 3.9 s (n=5). The EC_{50} for receptor activation by nicotine was 57 μM, and the Hill coefficient was 1.1 (data not shown). Currents induced by 100 μM nicotine were reversibly suppressed by 30 μM mecamylamine, a non-selective nAChR antagonist and by 20 nM methyllycaconitine (MLA), a specific α7 specific nAChR antagonist (Fig. 4B). Nicotine-induced inward current was reduced 85 ± 5% (n=4) by mecamylamine and 76 ± 7.6 % (n=4) by MLA. These studies show that H520 cells express both functional α7 nAChR and non-α7 containing nAChR. Recording from single cells showed that chronic exposure to nicotine resulted in increased activity of the nAChR expressed in the cultured SCC cells (Fig. 4C,D). Thus chronic nicotine exposure both upregulates and activates nAChR expression in SCC.

We have previously reported that M3 muscarinic antagonists can block SCLC growth by targeting MAPK proliferative pathways which are activated by both nicotinic and muscarinic cholinergic receptors. Given that SCC express similar nAChR and mAChR as SCLC, this suggests that M3 antagonists might similarly inhibit proliferation in SCC. H520 cells express functional mAChR as shown by the ability of atropine to block the ACh-induced increase in intracellular calcium (Fig. 5A). As shown in figure 5B the selective M3 mAChR antagonist darifenacin blocked the nicotine-induced increase in H520 cell proliferation *in vitro.* Darifenacin also significantly inhibited growth of H520 SCC xenografts in nude mice (Fig 5C,D). This suggests that SCC tumor growth can be blocked by targeting the activated cholinergic pathways present in SCC.

Discussion

Lung cancer expresses an intrinsic cholinergic signaling system such that exogenous nicotine and endogenous acetylcholine can stimulate tumor growth. As we show here the cholinergic system in SCC is upregulated at multiple levels. This upregulation combined with smoking by most lung cancer patients not only provides a considerable proliferative stimuli but also provides a pathway to target for new therapeutic approaches to lung cancer.

In early studies, Schuller et al (28) demonstrated that nicotine stimulated growth of lung cancer cell lines and Maneckjee and Minna (29) showed that nicotine blocked the inhibitory effect of opiates on lung cancer cell line growth. Subsequent studies have shown that nicotine acting through nAChR activates lung cancer growth through both the Akt and MAP kinase pathways (4–9). Similarly ACh acting through mAChR as well as nAChR has been shown to lead to cell proliferation by activation of MAP kinase (Erk1/2) and stimulation of cell cycle progression (17,18,30). Studies from our laboratory demonstrated that lung cancers express nAChR and mAChR as part of a stimulatory autocrine cholinergic pathway and that in addition to cholinergic receptors, lung cancers synthesize and secrete acetylcholine and express cholinesterases (11).

Squamous cell lung carcinomas are derived from bronchial epithelial cells. Thus, not surprisingly, normal bronchial epithelial cells also express a cholinergic autocrine loop (13). However, as shown in figures 1–3, cholinergic signaling is markedly upregulated in SCC compared to normal lung. As shown in figure 1A ChAT is strongly upregulated in SCC while cholinesterases are downregulated. This combination of increased synthesis and decreased degradation causes dramatic increases in ACh content of tumor compared to normal lung as shown in figure 3A. Thus SCC secrete increased levels of ACh to provide an endogenous proliferative stimuli to both mAChR and nAChR. The mechanism underlying the increased ChAT expression in SCC is not clear, though nicotine itself stimulates ACh secretion from H520 cells in culture (figure 3B). The observation of decreased cholinesterases in SCC are

consistent with the results of Martinez-Moreno et al (31) who reported decreases in AChE and BChE activity in both SCC and large cell carcinoma of the lung. The potential importance of decreased cholinesterase in tumor growth is further supported by Cabello et al (32) who showed that long term treatment of rats with cholinesterase inhibitors led to increased formation of mammary carcinomas that could be blocked by administration of the muscarinic antagonist atropine.

There is also a striking reduction in the levels of lynx1 in SCC (Fig. 1A). Lynx1 is a member of a newly described family of allosteric modulators of nicotinic receptor activity (33–35). Lynx1 has been shown to attenuate responses to ACh and to increase the extent to which ACh and nicotine desensitize nAChR (33). In brain, lynx1 knockout mice show dramatically increased responses to nicotine (36). Our laboratory has recently shown that Lynx1, the nAChR accessory protein is expressed in normal bronchial epithelium (25). We now find that lynx1 is widely expressed in SCC but is expressed at significantly lower levels in tumors than adjacent normal tissue. Thus decreases in lynx1 would be expected to potentiate SCC responses to exogenous nicotine and endogenous ACh.

As shown in figures 1 and 2, nicotinic receptors are expressed in the majority of SCC consistent with their expression in normal bronchial epithelium (25). Consistent with West et al (4), nAChR subunits including α3, α5, α7, α9, and β2 were found in most SCC (Figs. 1, 2). M2, M3 and M4 mAChR subtype were also found to be expressed in SCC (Figs. 1,2). These results imply that SCC might have homomeric nAChR containing α7, or α9 subunits and heteromeric forms containing both alpha and beta subunits. We did not check for α10, but given its wide expression in peripheral tissue it is also likely present in SCC and could form heteromers with α9 (37). There were no significant differences in levels of α3, α7, α9, and β2 nAChR subunits and M3 and M4 mAChR receptor between SCC and the adjacent normal tissues but levels of α5 and β3 nAChR subunits were significantly higher in tumor than normal tissues. The presence of increased levels of α5 and β3 subunits would suggest that growth of some SCC might be sensitive to mecamylamine which is a inhibitor of heteromeric nAChR subtypes. Consistent with this Zhu et al (38) has shown that mecamylamine inhibits second hand smoke stimulation of Lewis lung cancer cells in nude mice and we have previously shown that mecamylamine inhibits growth of SCLC cells *in vitro* (11). Interestingly levels of M2 receptors were decreased and we have previously reported that M2 activation appears to inhibit lung cancer growth (39).

Chi-Leung Lam et al (10) recently examined expression of nAChR in a series of NSCLC. Interestingly they did not see the increase in α5 and β3 we observed, but instead saw a small decrease in α4 and β4 which we did not observe. However their series was primarily adenocarcinomas with only 6 of 66 being SCC. In their individual results, levels of α4 and β4 did in fact appear higher in SCC than adenocarcinomas. Differences in receptor expression between lung adenocarcinomas and squamous cell carcinomas are to be expected since they derive from different lung cell types that also have different patterns of receptor expression. These differences show how important it is to fully characterize the different nAChR receptor expression by different lung cancer types as this may effect responses to therapy. Chi-Lueng Lam et al (10) also reported a striking difference in nAChR receptor expression between adenocarcinomas from smokers and non-smokers. SCC in never smokers is less common than adenocarcinoma in never smokers (3), so not surprisingly, none of the SCC in the series we studied came from non-smokers so we could not examine effects of smoking on receptor expression. In addition, we did not have pack year histories or gender identification associated with the tumors. It is quite possible that the number of pack years of exposure to exogenous nicotine might effect nAChR receptor expression patterns by the tumors. The role of gender on cholinergic expression patterns is also of great interest given the significantly higher frequency of lung adenocarcinomas in never smoker women than never smoker men (40).

What makes the expression of nAChR in lung cancer so significant is that the majority of patients are smokers and many continue to smoke even after diagnosis. Thus the nAChR in lung cancers are continually exposed to nicotine. The effect of nicotine on nicotinic receptor activity is highly complex and has been extensively studied in brain. In some neurons chronic nicotine exposure can upregulate and inactivate nAChR (41), in other systems chronic nicotine upregulates and activates nAChR (42). Nicotine has also been recently shown to act as a chaperone and increase targeting of mature nAChR in the cell membrane (43). As shown in figure 4A, nicotine increased expression of nAChR in H520 SCC cells. This is consistent with the finding of Chi-leung Lam et al (10) and consistent with our previous finding, that chronic nicotine increases nAChR expression in normal lung cells (15). Importantly, the upregulation of nAChR expression is associated with increased receptor activity as shown by whole cell voltage clamp recordings (Fig. 4). Antagonist studies show activity of both homomeric α7 nAChR and non-α7 containing heteromeric nAChR (Fig 4B).

In comparing levels of expression of components of the cholinergic signaling pathway between the normal and neoplastic lung samples, it is striking that all the significant changes were in the direction of increased cholinergic signaling in the tumors. First, levels of ACh increased in tumors reflecting increased ChAT and decreased cholinesterases expression. The increased levels of tumor ACh would then be able to stimulate proliferative pathways through both muscarinic and nicotinic mechanisms. Cholinergic stimulation of growth would likely be increased as levels of some nAChR subunits are increased, and levels of inhibitory M2 muscarinic receptors are decreased. Nicotinic receptors would also likely show less desensitization from endogenous ACh and exogenous nicotine as levels of lynx1 are decreased. Finally, we have shown that nicotine upregulates nAChR activity, so smoking will amplify responses to the increased levels of ACh found in lung cancers as well as amplify responses to continued smoking. Thus the changes seen in cholinergic signaling in SCC strongly support a role for nicotine to stimulate growth and progression of the tumors. Consistent with this, continued smoking has been shown to have a negative correlation on lung cancer survival (44–46). Therefore, in smokers with lung cancer, tumors would be stimulated both by endogenous ACh and exogenous nicotine. Each by itself is capable of stimulating proliferation of tumor cells and the combination of both has the potential to provide an additive stimuli to proliferation.

The mechanisms underlying the differences in cholinergic expression between tumors and normal lung remain to be determined and require further studies. The differences may derive from the cells of origin of SCC in that expression of nAChR, mAChR and ChAT is normally greater in bronchial epithelium than lung parenchyma. Alternately differences may reflect the effects of nicotine stimulating cholinergic expression in the tumor, reflect increased proliferation by the tumor or reflect other changes related to tumorigenesis. Finally it is possible that the differences could reflect sampling differences between normal and tumor tissue in which the normal samples do not represent a good sampling of normal large airway epithelium.

The upregulation of cholinergic signaling in SCC suggest that this pathway can be targeted for therapeutic intervention. A model of the cholinergic pathway is shown in figure 6, and as can be seen there are multiple potential targets. Dennis et al has shown that Akt pathway can be effectively used to block nicotine associated proliferation (4), and Trombino et al (47) has shown nAchR antagonists can inhibit growth of mesotheliomas. We have chosen to focus on the muscarinic receptor as a potential target since oral muscarinic antagonists are well tolerated and in wide clinical use for overactive bladder (48) and inhaled muscarinic antagonists are widely used for COPD (49). We have previously reported that M3 muscarinic antagonists can block growth of SCLC cell lines *in vitro* and in nude mice xenografts by preventing ACh and nicotine-induced increases in MAPK phosphorylation as well as lowering basal levels of

MAPK phosphorylation. Because of the inhibitory effects of M3 antagonists on SCLC proliferation we investigated their effects on proliferation of SCC.

As shown in figure 5A, the H520 line expresses muscarinic receptors as shown by the ability of the non-selective muscarinic antagonist atropine to block the ACh-induced increase in intracellular calcium. M3 selective antagonists similarly blocked the ACh-induced increase in intracellular calcium (data not shown). The M3 selective mAChR antagonist darifenacin also blocked the proliferative effect of nicotine on H520 cells (Figure 5B). This is consistent with our prior report that darifenacin blocks both basal and ACh-induced phosphorylation of MAPK (pERK 1/2) (22) and as shown in figure 6, MAPK activation represents an intersection of both nAChR and mAChR activation. Finally, as shown in figure 5C,D darifenacin also significantly inhibited growth of SCC cells both *in vitro* and in nude mice. This suggests that M3 selective antagonists may have clinical utility to slow the growth and progression of SCC by blocking the proliferative stimuli caused by endogenous and exogenous cholinergic stimulation. Given their widespread clinical use, it may be possible to derive epidemiologic data on lung cancer survival from individuals treated long term with M3 antagonists - though such studies have yet to be reported.

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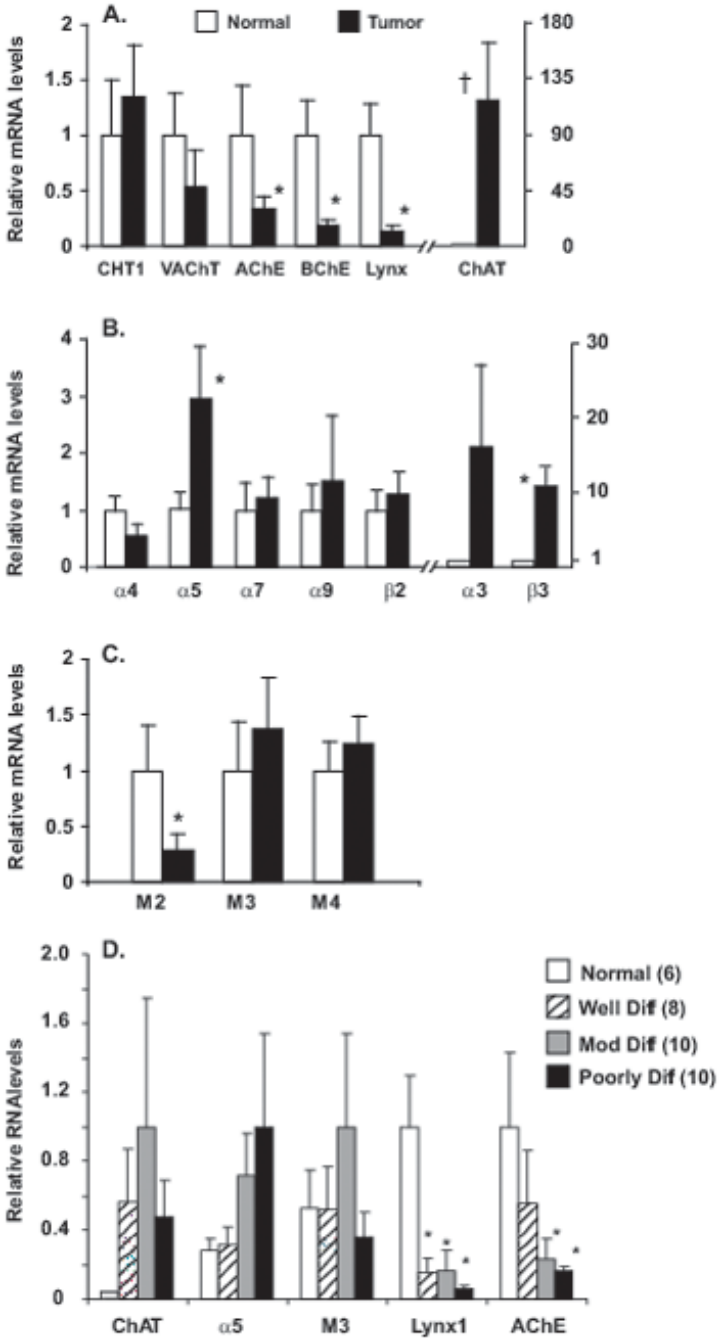

Figure 1.

Real-time PCR analysis of cholinergic mRNA expression in SCC tumor (solid bars) versus adjacent normal lung (open bars). Realtime was PCR performed as described in methods using 18S RNA as an internal standard. RNA levels were normalized to controls to allow comparison of fold change between tumors and normal. For ChAT, α3 and β3, fold change is plotted on right axes. **A**. Expression of ChT1, VAChT, AChE, BChE, lynx1 and ChAT mRNA in SCC and adjacent normal lung tissues. Expression of AChE, BChE and lynx1 was significantly lower in tumors than adjacent normal tissue (*, $p<0.03$); expression for ChAT was significantly higher in tumors than normal (†, $p < 0.01$). **B**. Expression of nAChR subunits in SCC. mRNA levels for α5 and β3 nAChR subunits was significantly higher in tumors than normal (*, p <

0.025) other differences were not significant. **C**. Expression of muscarinic receptors in SCC and adjacent normal tissue. mRNA levels for M2 mAChR were significantly decreased in tumors than normal (*, $p < 0.05$), levels of M3 and M4 were unchanged. Data are means ± SE;. n = 28 for tumors; n = 6 for adjacent normal lung tissues. **D.** Relative RNA levels of ChAT, α5 nAChR, M3 receptor, Lynx1 and AChE in squamous cell carcinomas plotted according to degree of differentiation. *$p<.05$ compared to normal tissue by Tukey-Kramer after 1 WAY ANOVA. Data for each RNA is normalized to 1.0 for the highest value within each RNA to allow comparison of changes within RNAs. Number of samples of each grade is shown in figure legend in parentheses.

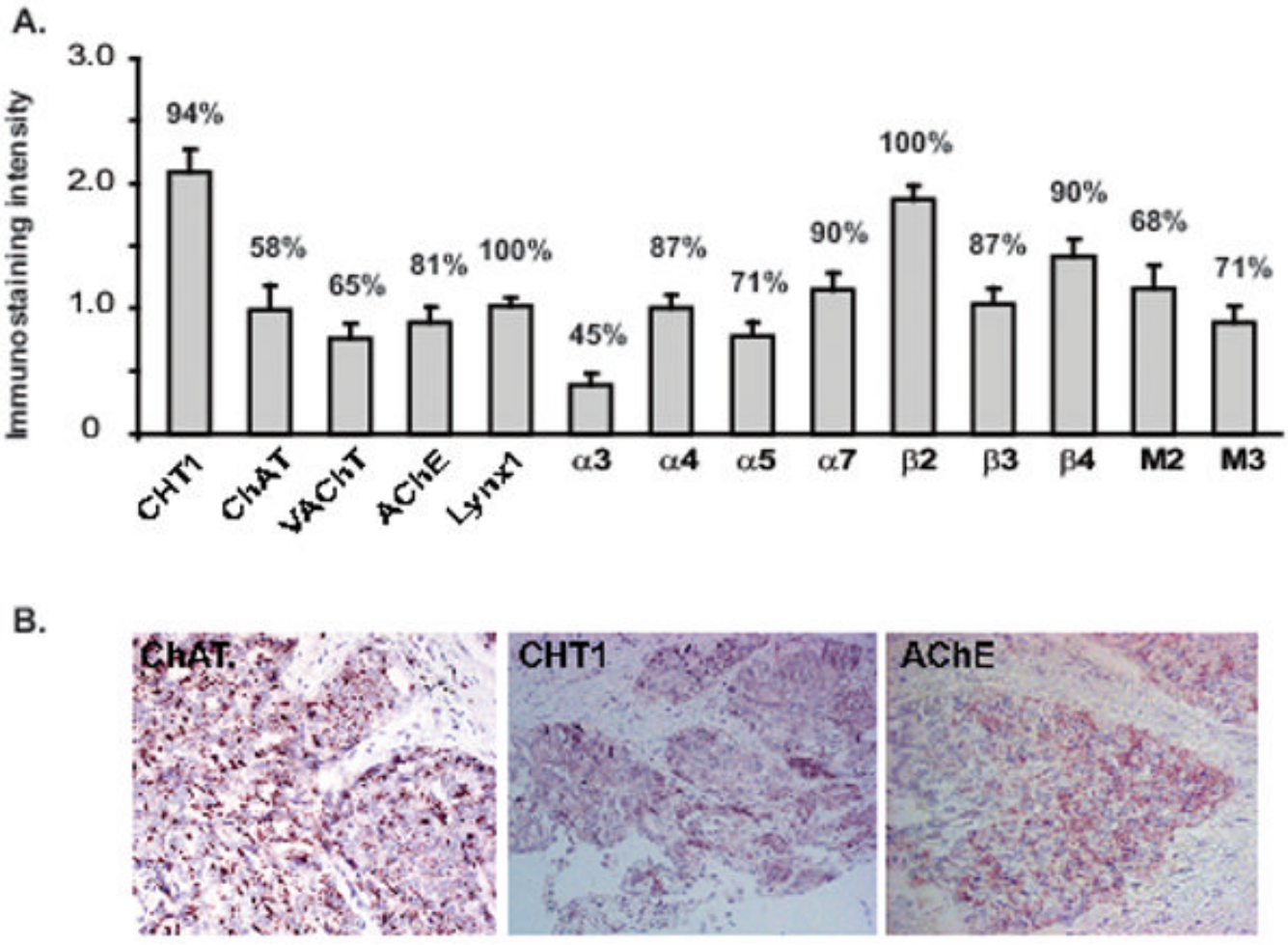

Figure 2.

A. Relative immunostaining for components of cholinergic signaling as shown. Intensity of staining scored from 0–4 as described in the methods and averaged for all samples. Percent of samples with positive staining shown above each bar. N = 31. **B.** Representative immunohistochemical staining of ChAT, CHT1 and AChE expression in SCC tumors (200X). Chromogen = VIP for all panels.

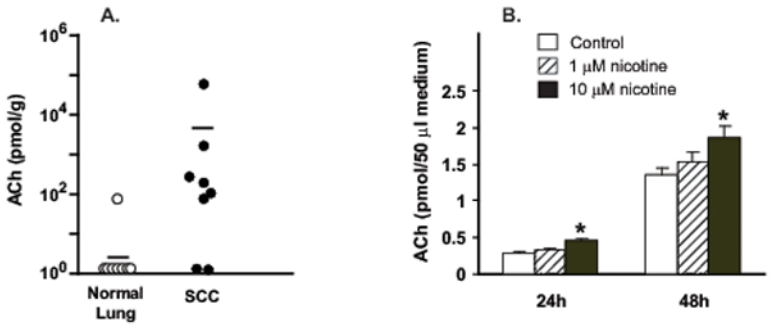

Figure 3.

ACh expression by SCC tumors, normal lung and cell lines. **A.** ACh content of normal lung and SCC tumors. ACh content of tumors expressed as pmol/g wet weight. ACh content of tumors was significantly higher than normal lung (note data plotted on log scale). **B.** Nicotine increased ACh secretion by H520 SCC cells in culture. 0.5 million H520 cells were plated in 24-well plates and incubated overnight. On the second day fresh media containing 50 μM neostigmine and nicotine at the concentrations shown was added. After an additional 24h or 48h incubation, ACh content in the media was measured. Data are mean ± SD of 4 experiments. * $p < 0.02$ compared to control.

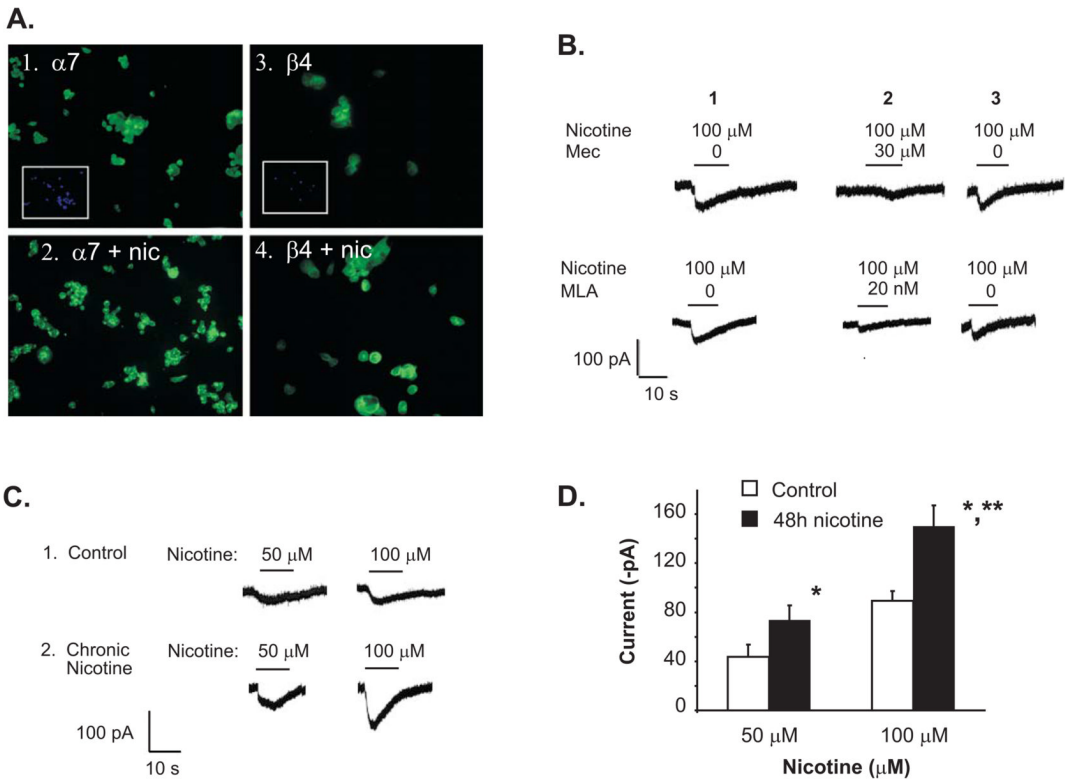

Figure 4.

Effect of nicotine on nAChR activity in SCC cells. **A.** Chronic nicotine exposure upregulates α7 and β4 nAChR immunostaining in H520 cells. Panel 1 shows control α7 nAChR immunostaining in H520 cells after 48 h incubation in medium alone, (inset picture is non-specific staining control). Panel 2 shows increased α7 immunostaining after 48 h incubation in medium + 2 μM nicotine. α7 nAChR staining increased by 29 ± 5 % (mean ± SE, n=3 experiments; one or two coverslips per experiment;15 fields of view per coverslip). Panel 3 shows control β4 nAChR staining; panel 4 shows increased staining after 48 h incubation in 2 μM nicotine. β4 nAChR staining increased by 24 ± 6 % (mean ± SE, n=3 experiments; one or two coverslips per experiment;15 fields of view per coverslip). **B.** Nicotinic receptor inward currents in H520 cells. Nicotine induces inward currents in H520 cells which are blocked by the nAChR antagonists mecamylamine and MLA. **C.** Chronic nicotine exposure upregulates responses to nicotine in H520 cells. Panel 1. Whole cell current in control H520 cells in response to application of nicotine by perfusion at concentrations shown (50, 100 μM). Panel 2. After 2 μM nicotine treatment for 48 hours, whole cell currents in response to application of nicotine by perfusion at concentrations shown (50, 100 μM). Holding potential was −60 mV. Experimental data were fitted by the Hill equation with EC_{50} = 57 μM and Hill coefficient = 1.1. **D.** Quantitation of increase in peak currents induced by nicotine after chronic nicotine exposure. * p< .01 for effect of nicotine by 2 way ANOVA, ** p < .05 compared to control by Tukey-Kramer multiple comparison test.

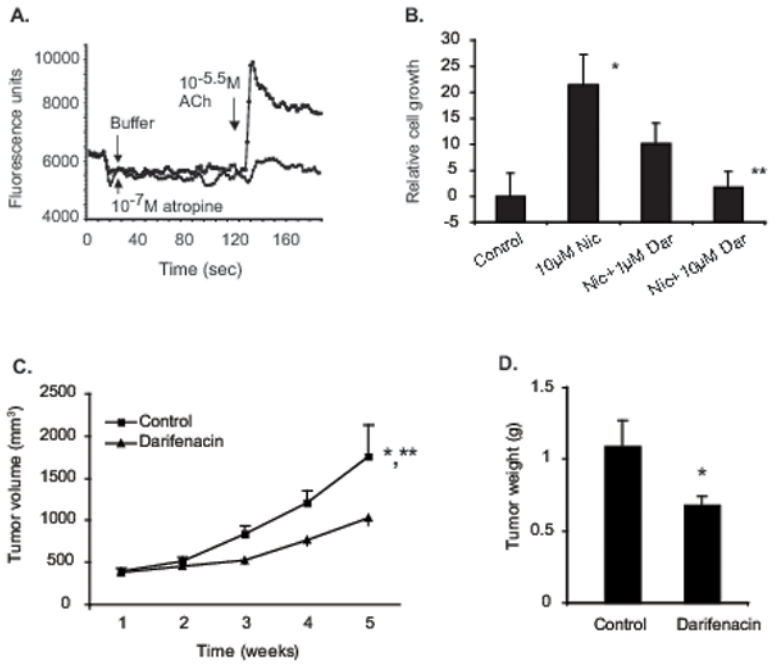

Figure 5.

Muscarinic antagonists inhibit cholinergic signaling and SCC cell growth. **A.** The muscarinic antagonist atropine inhibits the ACh-induced increase in intracellular calcium in H520 cells. **B.** The M3 muscarinic receptor darifenacin antagonist inhibits the nicotine-induced increase in H520 SCC cell growth. The data are the mean ± SE of two separate experiments, presented as % of increased cell proliferation in control. * P< 0.05 verus control; ** P< 0.05 verus 10 μM nicotine. **C**. Effect of darifenacin (6.0 mg/kg/day) on growth of H520 squamous cell lung carcinoma xenografts in nude mice. Tumor cells were injected on day 0 and darifenacin was started 1 week later. * $p < .001$ for overall effect of Darifenacin on tumor growth by 2 way ANOVA. ** $p < .05$ for treated versus control at 5 weeks by Tukey-Kramer multiple comparison test. **D**. Tumor weight at 5 weeks. * $p < .05$ compared to control by t-test.

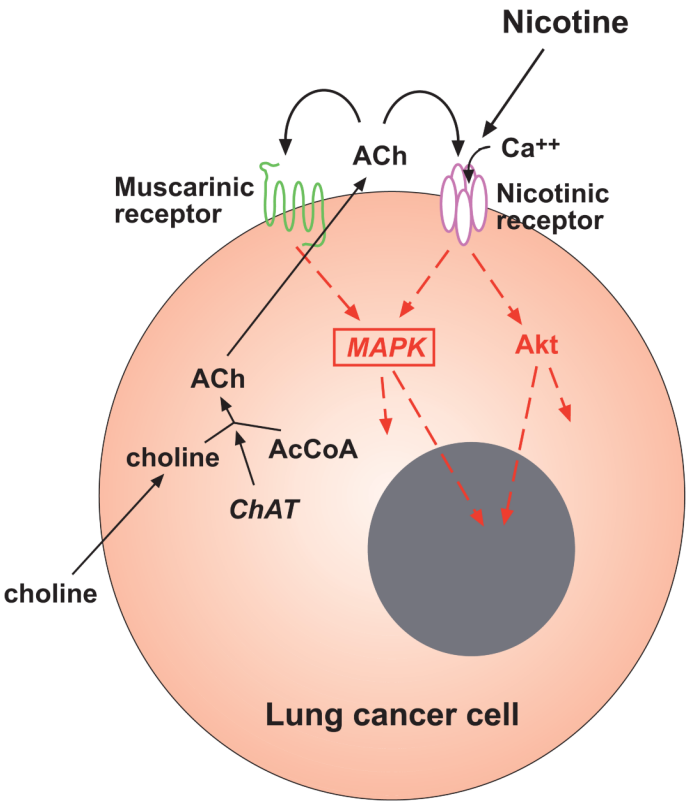

Figure 6.
Model of cholinergic signaling in SCC. Exogenous nicotine activates MAPK and Akt proliferative pathways through nicotinic cholinergic receptors. Endogenous ACh similarly activates both MAPK and Akt pathways through both muscarinic and nicotinic receptors. Thus muscarinic antagonists have potential to block proliferation induced both by exogenous nicotine and endogenous ACh as well as antagonizing the upregulation in cholinergic signaling induced by nicotine.