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Nicotinic acetylcholine receptor expression in human airway correlates with lung function

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Running head: nAChR expression in human airway related to lung function

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ABSTRACT

Nicotine and its derivatives, by binding to nicotinic acetylcholine receptors (*nAChR*s) on bronchial epithelial cells, can regulate cellular signaling and inflammatory processes. Delineation of *nAChR* subtypes and their responses to nicotine stimulation in bronchial epithelium may provide information for therapeutic targeting in smoking-related inflammation in the airway.

Expression of *nAChR* subunit genes in 60 bronchial epithelial biopsies and immunohistochemical staining for the subcellular locations of *nAChR* subunit expression were evaluated. Seven human bronchial epithelial cell lines (HBECs) were exposed to nicotine *in vitro* for their response in *nAChR* subunit gene expression to nicotine exposure and removal.

The relative normalized amount of expression of *nAChR* α4, α5 and α7 and immunohistochemical staining intensity of *nAChR* α4, α5 and β3 expression showed significant correlation with lung function parameters. Nicotine stimulation in HBECs resulted in transient increase in the levels of *nAChR* α5 and α6 but more sustained increase in *nAChR* α7 expression.

nAChR expression in bronchial epithelium was found to correlate with lung function. Nicotine exposure in HBECs resulted in both short and longer term responses in *nAChR* subunit gene expression. These results gave insight into the potential of targeting *nAChRs* for therapy in smoking-related inflammation in the airway.

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INTRODUCTION

Tobacco smoking is an important cause of lung function impairment, more commonly manifest as airflow obstruction on lung function tests. Nicotine in tobacco smoke is also one major component to cause damage to bronchial epithelium as well as being a potential carcinogen for the lung (12, 16). There were previous reports of nicotine stimulation of normal bronchial epithelium giving rise to aberrant *Akt* or NF-kB signaling that may contribute to the development of airway inflammation. Recent discoveries of functional acetylcholine receptors on lung epithelial cells and lung tumors raise the question of whether exposure to nicotine could participate in pathogenesis of airway disorders, one example would be chronic obstructive pulmonary disease (COPD). COPD is defined as an airway disorder resulting from chronic exposure to noxious gaseous substance like tobacco smoke and it usually manifests with respiratory symptoms together with the detection of non-reversible airflow obstruction on lung function tests (1). The ratio of forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC), i.e. FEV_1/FVC ratio of less than 70% is an indicator of airflow obstruction (1). Studying the pattern of nicotinic acetylcholine receptor (*nAChR*) expression in bronchial epithelium might provide information that nicotine is playing a role via specific subtypes of *nAChR*s in development of airflow obstruction.

nAChRs are found in non-neuronal tissues, such as α3, α5 and α7 in cultured bronchial epithelial cells (10, 22) but the pattern of *nAChR* expression is not completely delineated in human bronchial epithelial tissue. The mechanisms and contributions

towards development of lung disease by these various *nAChR* subunits are not well defined.

Recent meta-analysis has implicated the *CHRNA3-CHRNB4-CHRNA5* region on chromosome 15q25 to be associated with nicotine dependence, and this region is known to encode the different *nAChR* α subunits (21) (*CHRN* being the gene symbol for *nAChR*). In one study, genetic variants in this 15q24/25 region are associated with emphysema (8, 13), while large scale genome wide association study suggested that variants in that region could be genetic risk factor for the development of airflow obstruction independent of smoking (20). There is also evidence from Genome-Wide Association Study (GWAS) that these *nAChR* clusters may act on COPD development via susceptibility to smoking but not directly on development of COPD (2).

In this study, we aimed to delineate the expression pattern of *nAChR* subunits in bronchial epithelium, from normal looking areas at bronchoscopy with autofluorescence imaging (AFI), in correlation with clinical characteristics and lung function. Immortalized normal bronchial epithelial cell lines were established from the bronchial epithelial biopsy and were tested for *nAChR* expression in response to nicotine exposure for nine days. The results from these experiments would provide insight into the following scientific questions: What are the cellular locations for different *nAChR* subunit expression and what could be the anatomical and functional implications? Can these immortalized bronchial epithelial cell lines be models for further study of the functional role of *nAChR* in bronchial epithelium? Understanding the pattern and regulatory mechanisms of *nAChR* subunit expression in human airway may give insight into their roles in inflammatory airway disorders and relevant lung function changes.

MATERIALS AND METHODS

This was a prospective cohort study. Consecutive subjects, who have sputum atypia undergoing bronchoscopy with autofluorescence imaging (AFI) (Figure 1), were recruited. The inclusion criteria were sputum cytology examination demonstrating atypical cells but recent chest imaging did not reveal localizing lesion accountable for the sputum detection of atypical cells; and able to give informed written consent. The exclusion criteria were patients having active hemoptysis; or patients with bleeding tendency or on oral anticoagulation which may increase the risk of bleeding from bronchoscopic biopsies even if oral anticoagulation was stopped before bronchoscopy. Chest radiographs (CXRs) and computed tomography (CT) thorax scans were done in the six weeks before they were referred for AFI. Spirometry was performed three days before bronchoscopy. The study was approved by HKU/HKHA HKWC EC/IRB local Institutional Review Board (IRB) of the University of Hong Kong (HKU)/Hong Kong Hospital Authority Hong Kong West Cluster (HK HA HKWC 09-120) and the research has been carried out in accordance with the Declaration of Helsinki (2008). Non-smokers were patients who have never smoked for their life-time. Smokers included current smokers who have been current active chronic smokers and ex-smokers who have been daily smoking for more than twelve months in the past but have quit smoking for at least twelve months before bronchoscopy. Chronic smokers were asked to stop smoking, in particular on the day before lung function tests and bronchoscopy.

At bronchoscopy with AFI, suspicious areas will show up as areas of magenta fluorescence color, in contrast to the green fluorescence color for normal bronchial epithelium (Figure 1). After diagnostic specimens were biopsied from magenta areas, four additional bronchial biopsies would be taken from the adjacent green fluorescent areas for this study. The first piece of these four additional biopsies was cultured, while the next two pieces were paraffinized for histological sectioning, and the final one piece was saved in RNAlater solution (Qiagen, Hilden, Germany). Total RNA was extracted from the piece of bronchial biopsy kept in RNAlater solution. Lung function tests were performed with the Vmax Encore lung function test system (CareFusion, San Diego, CA, USA) and test results were interpreted according to the American Thoracic Society – European Respiratory Society recommendations (11).

Immortalized bronchial epithelial cell lines

Four immortalized bronchial epithelial cell lines, HBEC-KT 2 – 5, were used (from John Minna MD, University of Texas Southwestern Medical Center at Dallas, USA) (15). Three cell lines were established from recruited subjects with immortalization using the same laboratory protocol (15) and they were all beyond 100 passages at the time of these experiments. These new cell lines were named HKBS62N-KT, HKBS65.2N-KT and HKBS150N-KT.

Complementary DNA synthesis

Total RNA was extracted from tissue specimens and cell lines. RNA samples (1 μg) were reverse transcribed in 20 μl reaction mix (5x First-Strand Buffer [ThermoFisher Scientific, Waltham, MA, USA], DTT [100 mM, Promega, Madison, WI, USA], dNTP [1mM, Amersham Biosciences, Bath, UK], oligo-dT12-18 primers [ThermoFisher Scientific, Waltham, MA, USA] and random hexamer [Promega, Madison, WI, USA], RNaseOUT Recombinant Ribonuclease Inhibitor [ThermoFisher Scientific, Waltham, MA, USA] and Superscript II Reverse Transcriptase [ThermoFisher Scientific, Waltham, MA, USA]) with one-hour reaction at 42^{o}C.

Quantitative PCR reactions

The primers spanning across intron-exon junctions were designed for quantitative PCR targeting *nAChR* subunit genes α3, α4, α5, α6, α7, β2, β3and β4 with 18S as the reference gene. The methodology used was the same as described before (7). Quantitative PCR reactions were carried out in triplicates. SYBR Green I (SYBR Green JumpStart Taq ReadyMix [Sigma-Aldrich, Saint Louis, MO, UA]) was used as the detection dye and ribosomal 18S was used as the reference gene. Final reaction volume was 10 μl with 1.25 μl of 1/10 TE-diluted cDNA from RT reaction, 0.1 μM of each specific primer, 5 μl SYBR Green Jumpstart Taq ReadyMix. Quantitative PCR cycles were set at 10 min denaturation, followed by 40 cycles of 95^{o}C for 15s, 60^{o}C for 5s and 72^{o}C for 20s; and 72^{o}C for 10min as the final extension step. Dissociation curves were inspected for each pair of primers and only one dissociation peak must be present for each run of reaction before the results were considered to be valid. A five-fold serial dilution of a reference

sample was used for construction of standard curves with respect to each pair of primers. Quantitative PCR was run for each tumor or cell line cDNA sample and the Ct for a particular sample was obtained from the standard curves for a specific pair of primers. The Ct for unknown samples was compared with Ct of reference samples to obtain the Normalized Relative Amount for unknown samples. The Normalized Relative Amount was then $\log_2$ transformed and approximation to normal distribution estimated. For qPCR data, the Normalized Relative Amount for all samples was multiplied by a common factor of 1024 and then Log_2 transformed used for transformed qPCR data for direct visual inspection and comparison (7). The relative normalized amount of each *nAChR* subunit gene expression was correlated with lung function parameters.

Immunohistochemistry

nAChR antibodies (Santa Cruz, Dallas, Tx, USA) targeting different subunits α3, α4, α5, α6, α7, β2 and β3 were used for tissue sections of bronchial epithelium. The immunohistochemical staining intensity (Grade 0 – 3) of nAChR at different cellular locations (bronchial cell cytosol, bronchial gland serous membrane, bronchial gland mucus component) was scored by an independent pathologist (K-H Fu) who was blinded to the clinical information of all recruited subjects (Figure 2). The scored grading of each bronchial biopsy was correlated with lung function parameters.

Response to nicotine exposure in normal human bronchial epithelial cell lines

Seven NHBE cell lines, i.e. HBEC-KT 2 - 5 (15) and HKBS62N-KT, HKBS65N-KT and HKBS150N-KT, were cultured and equal number of passages for each cell lines were randomized into nicotine group and control group. The HBECs were seeded and incubated for 24 hours before addition of nicotine. On day 0, nicotine (100nM) was added to the nicotine group and the same volume of culture medium without nicotine was added to the control group. Nicotine at 100nM was found to be an easily achievable serum level in smokers (9). Both groups were further incubated for 9 days when the nicotine will be removed, with interim change of medium for once with nicotine medium replacement and plain medium without nicotine for each group. On day 9, both groups will switch to plain medium and were allowed to grow until day 12. Cells were harvested on days 0, 3, 6, 9, 12 days. Nine days were chosen as the usual population doubling time was around ten days for these cultured bronchial epithelial cells. Total RNA was extracted for reverse transcription and quantitative PCR as described above.

Statistical analysis

Student t tests, with assumptions of two-tails and unequal variance, were used for comparison of expression level of different subunit genes between groups of samples with different gender and smoking history. All expression levels were logarithmic transformed to achieve data normality. Chi-square tests were used for comparing the distribution of smokers and non-smokers with high or low mean expression of individual or combinations of *nAChR* subunit genes. Bonferroni adjustments were applied when multiple comparisons of the expression levels or immunohistochemical staining results

across different *nAChR* subunits. Statistical tests were carried out with IBM PASW for Windows version 21 (IBM, Armonk, New York, USA).

RESULTS

Demographics of recruited subjects

Seventy consecutive patients were referred for bronchoscopy with AFI for sputum atypia (Figure 1). Six declined participation in the study. Four subjects were not recruited because of recent treatment with oral anticoagulation. Sixty subjects were recruited with informed written consent obtained. There were 52 men and 8 women. The mean age was 61.8 ± 10.8 years. 32 of them were chronic smokers (53.3%), 19 were ex-smokers (31.7%) and 9 were non-smokers (15%). The mean amount of smoking was 24.9 ± 20.3 pack-years. The bronchial biopsies were reviewed by an independent pathologist (KHFu) who was not involved in subject recruitment and were blinded to their clinical characteristics. Out of the sixty bronchial biopsy specimens, 56 (93.3%) showed normal bronchial epithelium while two biopsies from smokers showed squamous metaplasia and two other biopsies from smokers showed moderate squamous dysplasia. The demographic characteristics of these lung cancer patients were summarized in Table 1. These recruited subjects were not diagnosed to have COPD before and they have no significant respiratory symptoms. Airflow obstruction (defined as $FEV_1/FVC < 70\%$ on lung function tests)(1) was found in 37 (61.7%) of subjects with FEV_1/FVC of 65.8 ± 12.9%

most of which were considered mild degree of airflow obstruction with FEV_1 (% of predicted) at 87 ± 17.9%. CT scan of thorax for all the subjects showed no abnormalities.

Quantitative PCR analysis of nAChR subunit gene expression

The relative normalized amount of *nAChR* mRNA expression of *nAChR* α4, α5 and α7 showed significant correlation with lung function, adjusted for gender and smoking (Table 2). The mRNA expression level of *nAChR* α4 was found to correlate with most spirometry parameters of FEV_1 (r = 0.325, p = 0.023) (Figure 3A), FEV_1 (% pred) (r = 0.375, p = 0.010), FVC (r = 0.485, p = 0.041), FVC (% pred) (r = 0.593, p = 0.022). The same correlation patterns were found for *nAChR* α5 mRNA expression level, correlating with FEV_1 (r = 0.338, p = 0.049)(Figure 3B), FEV_1 (% pred) (r = 0.403, p = 0.026), FVC (r = 0.469, p = 0.024), FVC (% pred) (r = 0.587, p = 0.011), and for *nAChR* α7 mRNA correlating with FEV_1 (r = 0.370, p = 0.034)(Figure 3C), FEV_1 (% pred) (r = 0.425, p = 0.011), FVC (% pred) (r = 0.576, p = 0.048). The *nAChR* β2 mRNA was found to correlate with FEV_1/FVC ratio (r = -0.219, p = 0.011).

Immunohistochemical staining

Out of sixty subjects recruited, 39 endobronchial biopsies were found to be of sufficient evaluable quality after histological examination to proceed on to immunohistochemistry. The age and gender distribution, smoking status and lung function parameters of these 39 subjects were found to be similar and not significantly different from those characteristics

of the whole group of 60 recruited subjects (Table 1). The staining intensity of *nAChR* α4 bc (surface epithelial cytosol staining), α5 sc (bronchial gland serous cell cytosol staining) and β3 mc (bronchial gland mucous cell staining) intensity (Figure 2) was found to correlate with lung function parameters. The *nAChR* α4 bc staining intensity was found to correlate with FVC (% pred) ($r = 0.552$, $p = 0.025$), while *nAChR* α5 sc staining intensity was found to correlate with FEV_1 (% pred) ($r = 0.369$, $p = 0.026$) and *nAChR* β3 mc staining intensity correlated with FEV_1 ($r = 0.367$, $p = 0.035$), adjusted for the effects of age, gender and smoking status of included subjects (Table 3).

Correlations of nAChR subunit gene mRNA expression levels with immunohistochemical staining intensity

The mRNA expression levels of *nAChR* subunit gene α4, α5, α7 and β2 showed significant and highest correlation coefficient with the corresponding immunohistochemical staining intensity (Table 4). There were also significant but weaker correlations between the mRNA expression levels with immunohistochemical staining intensity for *nAChR* α4, α5, α6 and α7 (Table 4), and the significance remains after adjustment for multiple comparisons.

Exposure of normal human bronchial epithelial cell lines to nicotine

When HBEC lines were exposed to 100 nM nicotine and then RNA harvested at days 0, 3, 6, 9 and 12, a significant rise in the expression levels of *CHRN* A5 and A7 were found

with rapid return to baseline levels of expression upon nicotine removal (Figures 4 A and 4C). There was a sharp rise in *nAChR* α5 subunit gene expression upon exposure to nicotine, and a similar sharp but slightly delayed rise in *nAChR* α7 subunit gene expression. Both these responses dropped with withdrawal of nicotine on day nine (Figures 4A and 4C). There was also a sharp but not sustained rise in *nAChR* α6 subunit upon nicotine exposure and it dropped after day 6 before nicotine was removed on day 9 (Figure 4B). No significant rise or fall was found in the levels of expression of other *nAChR* subunit genes including *CHRN* A3, A4, B2, B3 and B4 analysed.

DISCUSSION

The distribution, function and ligand-binding affinity of nAChR depends on the composition of nAChR subunits, although the exact function and physiological roles of individual nAChR subtypes are not completely understood. To our knowledge, the correlation of expression of these *nAChR* subunits with lung function has not been reported before. The quantitative PCR analysis of *nAChR* subunit gene expression levels allowed for quantitative comparison between groups of samples with respect to gender and smoking habits.

In this study the most significant correlations between lung function parameters and *nAChR* α4, α5 and α7 expression levels or the corresponding immunohistochemical staining intensity of each nAChR subunit expression. *CHRNA5* is known to be associated with nicotine dependence (21) and the presence of emphysema in smokers (8, 13). The result of this study provided direct support to previous GWAS study that *CHRNA5* expression was linked to the development of airflow obstruction (20) independent of effects of age, gender and smoking status. The subjects included in this subjects were either having no airflow obstruction or just mild degree of it (FEV_1 [% of predicted value] between 50 – 80%). The presence of *nAChR* α5 subunit in *nAChR* has been shown to alter the calcium permeability and nicotine sensitivity in vitro (5). *nAChR* α7 subunit was found to be important in the control of nicotine-induced calcium-influx in SCLC (17) and was thus thought to be important in growth signal transduction induced by nicotine binding to nAChR. The expression of *nAChR* α5 and α7 in normal bronchial epithelium suggested that they may play similar roles in nAChR signaling and such correlation with airflow parameters on lung function tests would suggest that there could be activated nAChR signaling in response to nicotine in smokers correlating with airflow obstruction.

Although we have found significant reduction in *nAChR* α4 expression in lung cancer (7), there is so far no further evaluation of its role in normal bronchial epithelium. There is recent evidence that nAChR α7 and α4β2 activation (16) could be associated with transactivation via cyclic adenosine monophosphate (cAMP) stimulation of the epidermal growth factor receptor (*EGFR*) signaling pathways inducing cell growth and proliferation, thus may contribute to carcinogenesis (16). This cAMP activation could also be potential target for phosphodiesterase inhibition, which is one therapeutic strategy in targeting inflammation in COPD (1). The transient but not sustainable *nAChR* α6 that dropped before nicotine withdrawal was also intriguing and deserves further investigation into the roles of *nAChR* α6 in normal bronchial epithelial cells.

CHRNA5 and *A7* showed significant reversible induction of expression on *in vitro* exposure to nicotine, providing direct evidence that these subunit genes respond to acute nicotine exposure and could mediate the immediate or short term effects of nicotine. Little is known about the functional significance of the expression these specific *CHRNA5* and *A7* in the central nervous system or in lung cancer, though *CHRNA7* had been reported to be expressed in SCLC cell lines (18) and was thought to be related to smoking in schizophrenic patients (4). Up-regulation of functional *CHRNA7* has also been demonstrated in normal human bronchial epithelial cells on exposure to nicotine (19). The return of the expression levels of those three subunits, of *nAChR* α5, α6 and α7, to baseline upon cessation of nicotine exposure may reflect that continued or chronic exposure to nicotine (usually taken to be more than 10 days) of continuous exposure to nicotine (14) was required for nicotine addiction or other cellular effects of nicotine mediated by specific *nAChR* subunits. The effects of chronic exposure to nicotine on *nAChR* subunit gene expression in these normal human bronchial epithelial cells and whether the

same response is maintained, or other *nAChR* subunit genes would be involved, in chronic nicotine exposure warrant further evaluation.

In neural tissues, the up-regulation of *nAChR*s in response to chronic nicotine is consistently reported as post-translational events without changes in mRNA (3, 6). In this study, we have demonstrated nicotine induced increases in the expression levels of both *nAChR* mRNA and protein in culture bronchial epithelial cells, nicotine-induced up-regulation of nicotinic receptors in bronchial epithelial cells may include both transcriptional and post-translational mechanisms.

Although this is a small sample size, it did show correlation between different *nAChR* subunits in terms of mRNA expression level and the protein expression level by immunohistochemical staining. *nAChR* expression was correlated with lung function parameters that are suggestive of airflow obstruction. The other parts of lung function tests including lung capacity assessment and diffusion capacity measurements were not performed in this study. This supported our initial hypothesis of correlations between *nAChR* expression and lung functions, contributing to the development of airflow obstruction which could be one characteristic of smokers with COPD. Validation in a larger cohort of smoker and non-smoker subjects would be warranted but this initial sample of 60 apparently healthy subjects were already difficult to recruit. The chance of validation in COPD subjects would be even more difficult as there is seldom indication for bronchoscopic biopsy or surgical biopsy in COPD subjects, adding to the difficulties in performing similar clinical studies. The functional roles of different nAChR subunits, working on individual basis or in combination, in bronchial epithelium and how their expression levels translate into lung function abnormalities and ultimately clinical airflow obstruction requires further work.

In summary, there are correlations between *nAChR* subunit expression in the bronchial epithelium with lung function parameters. These *nAChR* subunit genes could be contributing to the development of airflow obstruction in smokers. Further evaluation of the functions and roles played by these *nAChR* subunit genes in the development of tobacco-related airway disorders are warranted.

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AUTHOR DISCLOSURES

All authors in this manuscript declared no conflict of interest.

Roles of each author:

David CL Lam (DCL) was involved in the overall supervision of laboratory experiments, lung function tests, clinical data collection and recruitment of bronchoscopy, data analysis and drafting of this manuscript

Susan Y Luo (SYL) carried out the real-time PCR and immunohistochemistry experiments.

Kin-Hang Fu (KHF) reviewed and scored immunohistochemical staining results.

Macy MS Lui (MML) supervised data analysis and helped in drafting manuscript

Koon-Ho Chan (KHC) supervised laboratory experiments

Ignacio I Wistuba (IIW) reviewed histology and immunohistochemistry experiments

Boning Gao (BG) contributed to the immortalization of normal bronchial epithelial cells and was involved in the in vitro testing with nicotine.

Sai-Wah Tsao (SWT) provided expertise advice on design of laboratory experiments and reviewed laboratory results and drafted this manuscript

Mary SM Ip (MSI) provided expertise advice on correlation and analysis of laboratory results with lung function and drafted this manuscript

John D Minna (JDM) inspired the overall conception of the project and provided expertise advice on experiment work and drafted this manuscript.

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Legends for Figures and Tables

Table 1
Basic demographics of all the 60 recruited subjects and the corresponding demographics of 39 subjects with biopsies evaluable by immunohistochemistry (IHC evaluable) are also listed in parallel

Table 2
Partial correlations of relative normalized amount of each *CHRN* subunit gene mRNA expression levels with lung function parameters

Table 3
Correlations of expression level of different *nAChR* by immunohistochemical intensity with lung function parameters.

Table 4
Correlation matrix of *nAChR* mRNA expression levels with immunohistochemical intensity.

Figure 1
Side by side comparison of endoscopic view with (a) and (b) showing ordinary white-light bronchoscopy (WLB) and the corresponding view under autofluorescence imaging (AFI), with the green fluorescence in AFI representing normal bronchial epithelium while the magenta areas (arrows) indicating the potential sites of abnormalities not visualized on the corresponding WLB views. Bronchial biopsies for this study were taken away from AFI magenta areas where the areas appeared green in fluorescence under AFI.

Figure 2
(A) Immunostaining pattern of bronchial epithelial cells. Apical cell membrane (bm), arrow heads and cytosol (bc), long arrows. There is accentuation of the staining at the apical cell membrane and terminal web (arrows) of the ciliated bronchial cells compared with the cytosol. Immunostain for nAChRs α4, Nikon Eclipse Ni-U with Plan Achromat 40x objective, c-mount 0.7x and 2/3" CCD camera with 5 mega pixels.

(B) Immunostaining pattern of bronchial gland acini. Serous cells (sc), arrowheads and mucous cells (mc), long arrows. The staining reaction is seen mainly within the cytosol. Immunostain for nAChRs α3, Nikon Eclipse Ni-U with Plan Achromat 20x objective, c-mount 0.7x and 2/3" CCD camera with 5 mega pixels.

Figure 3 (A – C)
Scatter plots showing significant correlations of FEV_1 with relative normalized amount of (A) *CHRNA4*; (B) *CHRNA5* and (C) *CHRNA7* mRNA expression level.

Figure 4 (A – C)
Graphic representations of the results of nicotine exposure for nine days in immortalized bronchial epithelial cells (with the red series summarizing the mean +/- SE of cell lines exposed to nicotine [treatment group] and with the blue series representing the respective

cell lines without exposure to nicotine[control group]) showing (A) sharp rise in *nAChR* α5 subunit gene expression upon exposure to nicotine, with a similar sharp but (C) a slightly delayed rise in *nAChR* α7 subunit gene expression. Both these responses dropped with withdrawal of nicotine on day nine. (B) There was also a sharp but not sustained rise in *nAChR* α6 subunit upon nicotine exposure but it dropped after day 6 before nicotine was removed on day 9.

Table 1

N	**60** (Total)	**39** (IHC evaluable)
Gender (F:M)	8:52	4:35
F	8 (13.3%)	4 (10.3%)
M	52 (86.7%)	35 (89.7%)
Mean Age (years +/- SD)	61.8 ± 10.8	63.1 ± 9.9
Range	30-83	33 – 79
Smoking status (NS:CS:EX)		
NS	9 (15.0%)	6 (15.3%)
CS	32 (53.3%)	19 (48.7%)
EX	19 (31.7%)	14 (35.9%)
Amount of smoking (pack-year)	24.9 ± 20.3	22.1 ± 18.6
FEV1/FVC (%)	65.8 ± 12.9	68.7 ± 10.1
Without AFO	23 (38.3%)	14 (35.9%)
With AFO	37 (61.7%)	25 (64.1%)
FEV_1 (L)	2.01 ± 0.56	2.63 ± 0.49
FEV_1 (% pred)	87.0 ± 17.9	88.3 ± 15.4
Mild (>80%)	41 (68.3%)	28 (71.8%)
Moderate (50 – 80%)	19 (31.7%)	11 (28.2%)
FVC (L)	3.21 ± 0.73	3.44 ± 0.66
FVC (% pred)	102.5 ± 15.2	99.8 ± 13.3
Histology		
No abnormality detected	56 (93.3%)	35 (89.8%)
Moderate squamous	2 (3.4%)	2 (5.1%)
Squamous metaplasia	2 (3.4%)	2 (5.1%)

NS = non-smoker, CS = chronic smoker, EX = ex-smoker;
FEV_1 = forced expiratory volume in one second, FVC = forced vital capacity; AFO = airflow obstruction; L = liter; % pred = percentage of predicted values

Table 2

	CHRNA3	***CHRNA4***	***CHRNA5***	***CHRNA6***	***CHRNA7***	***CHRNB2***	***CHRNB3***	***CHRNB4***
FEV_1	0.019 (p=0.587)	**0.325* (p=0.014*)**	**0.338* (p=0.010*)**	-0.002 (p=0.059)	**0.370* (p=0.004*)**	-0.061 (p=0.302)	-0.108 (p=0.833)	-0.053 (p=0.263)
FEV_1 (% pred)	0.040 (p=0.355)	**0.375* (p=0.043*)**	**0.403* (p=0.011*)**	-0.071 (p=0.071)	**0.425* (p=0.023*)**	-0.080 (p=0.456)	-0.181 (p=0.111)	-0.126 (p=0.112)
FVC	0.038 (p=0.217)	**0.485* (p=0.041*)**	**0.469* (p=0.032*)**	-0.144 (p=0.231)	0.471 (p=0.054)	-0.179 (p=0.125)	-0.050 (p=0.542)	-0.061 (p=0.255)
FVC (% pred)	0.086 (p=0.116)	**0.593* (p=0.021*)**	**0.587* (p=0.011*)**	-0.033 (p=0.067)	**0.576* (p=0.013*)**	-0.211 (p=0.433)	-0.119 (p=0.113)	-0.234 (p=0.132)
FEV_1/FVC	0.154 (p=0.213)	0.037 (p=0.313)	0.056 (p=0.543)	-0.120 (p=0.121)	0.076 (p=0.073)	**-0.419* (p=0.043*)**	-0.079 (p=0.217)	-0.072 (p=0.562)

FEV_1 = forced expiratory volume in one second, FVC = forced vital capacity; r values are shown correlating lung function parameters with quantitative expression of various nAChRs. Significant p values (* $p < 0.05$) are underlined in bold.

Table 3

Staining by cellular location: bm = surface epithelial cell membrane; bc = surface epithelial cytosol; dm = bronchial gland duct membrane; dc = bronchial gland duct cytosol; sm = bronchial gland serous cell membrane; sc = bronchial gland serous cell cytosol; mm = bronchial gland mucous cell membrane; mc = bronchial gland mucous cell cytosol; FEV_1 = forced expiratory volume in one second, FVC = forced vital capacity; r values are shown with significant values (* $p < 0.05$) indicated.

	bm	bc	dm	dc	sm	sc	mm	mc
nAChR α4								
FVC (% Pred)	-0.439	0.552*	0.019	-0.025	0.363	0.180	0.363	0.123
nAChR α5								
FEV_1 (% Pred)	0.121	0.066	0.073	-0.295	0.077	0.369*	0.014	0.244
nAChR β3								
FEV_1	0.177	0.210	-0.088	-0.270	-0.148	0.317	-0.081	0.367*

Table 4

Immunohistochemical Expression

mRNA expression	***CHRNA3***	***CHRNA4***	***CHRNA5***	***CHRNA6***	***CHRNA7***	***CHRNB2***	***CHRNB3***
α3	0.067	0.161	0.183	0.086	0.132	0.150	0.292
α4	0.254	**0.579***	**0.359***	0.141	**0.401***	0.175	0.126
α5	0.063	**0.373***	**0.595***	0.079	**0.433***	0.149	0.234
α6	0.139	**0.500***	**0.360***	0.090	0.252	0.197	0.293
α7	0.083	**0.366***	0.194	0.082	**0.552***	0.152	0.227
β2	0.067	0.139	0.138	0.029	0.031	**0.531***	0.253
β3	0.093	0.128	0.051	0.003	0.118	0.277	0.076

The header row lists the mRNA expression of different *nAChR* subunit correlating with the corresponding *nAChR* subunit detection by immunohistochemistry in each column. Significant correlations are in bold (*$p < 0.05$).