CHR\textsubscript{NA5} as negative regulator of nicotine signaling in normal and cancer bronchial cells: effects on motility, migration and p63 expression

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Genome-wide association studies have linked lung cancer risk with a region of chromosome 15q25.1 containing \textit{CHRNA3}, \textit{CHRNA5} and \textit{CHRNA4} encoding \(\alpha_3\), \(\alpha_5\) and \(\beta_4\) subunits of nicotinic acetylcholine receptors (nAChR), respectively. One of the strongest associations was observed for a non-silent single-nucleotide polymorphism at codon 398 in \textit{CHRNA5}. Here, we have used pharmacological (antagonists) or genetic (RNA interference) interventions to modulate the activity of \textit{CHRNA5} in non-transformed bronchial cells and in lung cancer cell lines. In both cell types, silencing \textit{CHRNA5} or inhibiting receptors containing nAChR \(\alpha_5\) with \(\alpha\)-conotoxin MII exerted a nicotine-like effect, with increased motility and invasiveness \textit{in vitro} and increasing calcium influx. The effects on motility were enhanced by addition of nicotine but blocked by inhibiting \textit{CHRNA7}, which encodes the homopentameric receptor \(\omega_7\) subunit. Silencing \textit{CHRNA5} also decreased the expression of cell adhesion molecules P120 and ZO-1 in lung cancer cells as well as the expression of Delta\textsubscript{N}p63\textsubscript{\alpha} in squamous cell carcinoma cell lines. These results demonstrate a role for \textit{CHRNA5} in modulating adhesion and motility in bronchial cells, as well as in regulating p63, a potential oncogene in squamous cell carcinoma.

**Introduction**

Lung cancer is the most frequent cancer among men in many countries and represents the primary cause of cancer death worldwide. In Europe, it accounts for an estimated 268,600 annual new cases among men and 82,900 cases among women (1). Smoking is the main risk factor worldwide. The cumulative risk by age 75 of continuous smokers is in the order of 15–20%, versus ~1% in never smokers (2). In 2008, three independent genome-wide association studies identified a lung cancer susceptibility region on chromosome 15q25.1, with a consistent measure of effect between the studies (OR = 1.30, \(P = 5 \times 10^{-20}\); OR = 1.31, \(P = 1.5 \times 10^{-16}\); OR = 1.32, \(P = 1 \times 10^{-17}\); OR, odds ratio) (3–5). This region encompasses three cholinergic nicotinic receptor genes (\textit{CHRNA3}, \textit{CHRNA5} and \textit{CHRNA4}) encoding nicotinic acetylcholine receptor (nAChRs) subunits \(\alpha_3\), \(\alpha_5\) and \(\beta_4\) in neuronal and other tissues. Given the frequency of at risk genotypes in the population, this association is responsible for up to 14% of lung cancer cases irrespective of histological subtype (4). In 2009, the same \textit{CHRNA} locus was identified as associated with chronic obstructive pulmonary disease (6).

**Abbreviations:** BrdU, 5-bromo-2′-deoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; nAChR, nicotinic acetylcholine receptor; NHBE, normal human bronchial epithelial cells; OR, odds ratio; PBS, phosphate-buffered saline; SNP, single-nucleotide polymorphism; siRNA, small interfering RNA; TDC, three-dimensional cultures.

The strong association with \textit{CHRNA} 3/5 locus has led to suggestions that nAChR variants may increase lung cancer risk indirectly of tobacco addiction. In one of the genome-wide association studies, an association was identified between the same genetic region and smoking quantity (5). However, recent studies in Asian populations in which never smokers are better represented have reported a similar association among never and ever smokers, as well as among men and women (7). These results suggest that the 15q25.1 region has an independent effect on lung cancer risk above that of its effect on smoking propensity.

One of the strongest associations within the 15q25.1 region is observed for the non-silent single-nucleotide polymorphism (SNP) rs16969968 in \textit{CHRNA5}, a G-to-A substitution encoding either aspartic acid (D) or asparagine (N) at codon 398. This residue is located in the second intracellular loop of the four-transmembrane structure of the nAChR \(\alpha_5\) subunit. SNP rs16969968 is in linkage disequilibrium with a non-coding SNP in \textit{CHRNA3} (4). The functional impact of these SNPs is unknown. nAChRs are ionotropic ligand-activated neurotransmitter receptors expressed by neuronal and non-neuronal cells throughout the body [reviewed in ref. (8)]. They form pentameric protein complexes activated in response to the endogenous neurotransmitter acetylcholine or to exogenous agonists, such as nicotine or tobacco-specific nitrosamines NNK or NNK (9–11). Nicotine mimics the effect of acetylcholine by inducing a conformational change at the internal side of the membrane that allows exchange of cations (Ca\textsuperscript{2+}, Na\textsuperscript{+}, K\textsuperscript{+}) between extracellular space and cytoplasm. In turn, Ca\textsuperscript{2+} influx opens the gates of voltage-activated calcium channels, further increasing intracellular Ca\textsuperscript{2+} with pleiotropic effects on pathways controlling cell motility, proliferation, differentiation and apoptosis (8,12,13). NACHRs are expressed in most epithelial cells including the bronchial epithelium and lung cancer cells (14–16). Six different receptor subunits have been detected in lung cancers, \(\alpha_3\), \(\alpha_4\), \(\alpha_5\), \(\alpha_7\), \(\beta_2\) and \(\beta_4\). The main receptor forms in bronchial cells are homopentameric \(\alpha_7\) receptors and heteropentameric receptors, such as the (\(\alpha_3\beta_2\)) \(\alpha_5\) receptor (16,17).

Signaling through nAChRs modulates motility and migration in several types of cultured epithelial cells including bronchial cells. While the proliferative effects of nicotine in these cells appear to be mediated through homopentameric \(\alpha_7\) receptors, heteropentameric (\(\alpha_3\beta_2\)) \(\alpha_5\) receptors contribute to cell motility and wound healing of the respiratory epithelium by modulating intracellular calcium influx in migrating cells (16,17). To better understand the potential impact of \textit{CHRNA5} in susceptibility to lung cancer, we investigated the role of this subunit on migration, adhesion and calcium influx in non-transformed bronchial cells and in lung cancer cell lines.

**Materials and methods**

**Cell lines, drug treatment and transfections**

Human tracheal epithelial SV40-transformed cells (16HBE) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% of fetal bovine serum, 1% of glutamine and 1% of antibiotics. Normal human bronchial epithelial cells (NHBE) were purchased from Lonza (Walkersville, MD) and used at passages 2–3. They were grown in bronchial epithelial cell basal medium (Lonza) supplemented with 0.4% bovine pituitary extract, 0.1% hydrocortisone, 0.1% human epidermal growth factor, 0.1% epinephrine, 0.1% transferrin, 0.1% insulin, 0.1% retinoic acid, 0.1% triiodothyronine, 0.1% gentamicin/ampicillin B (GA-1000) (Lonza). The human cancer cell lines A549 (lung carcinoma), H1299 (carcinoma, non-small-cell lung cancer), TE1 and TE13 (esophageal squamous cell carcinoma) were used. H1299, TE1 and TE13 were cultured at 37°C and 10% CO\textsubscript{2} in RPMI 1640 (PAA Laboratories GmbH, Pasching, Austria) medium and A549 at 37°C and 5% CO\textsubscript{2} in DMEM medium (PAA Laboratories GmbH). Both media were supplemented with 10% of fetal bovine serum, 1% of glutamine (Sigma, Saint Quentin Fallavier, France) and 1% of antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Sigma).

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Cells were seeded 24 h prior to treatment by nicotine (1 μM) (Sigma), 2-β-bungarotoxin (1 μM) (Toxic Bioscience, Ellenville, NY) or zα4-30ntoxin MII (10 nM) (Toxic Bioscience) for 2 h. Transfections of small interfering RNA (siRNA) CHRNA5 (L-006137-00-00, Thermo Scientific, Rockford, IL), siRNA CHRNA4 (ID 118985, Applied Biosystems, Foster City, CA) and siRNA CHRN1 (ID 7044, Applied Biosystems) were performed with HiPerfect transfection reagent (Qiagen, Hilden, Germany) in optiMEM (Gibco, Gibco- BRL, Invitrogen, Carlsbad, CA) and 2.5 nM siRNA (A549, TE1, TE13 cells) or 7.5 nM siRNA (H1299 cells). As a control, a generic scramble sequence was used (silencer negative control #2 siRNA—ID AM4613, Applied Biosystems). For 48 h, the medium was removed, a second transfection was performed to enhance gene silencing and cells were further incubated for 24 h prior to analysis.

**Migration assay**

Measurement of cell migration through microporous membranes coated with BD Matrigel™ (Matrigel invasion chambers, 354480; BD Biosciences, Bedford, MA) was performed using a Boyden Chamber assay. The membranes were rehydrated for 2 h in serum-free medium at 37°C. The lower chamber was filled with 750 µl serum-containing medium and the upper chamber with 350 µl of serum-free medium. Drugs were added to the upper chamber. For NIHBE cells, we used a bronchial epithelial cell basal medium depleted of supplements in the upper chamber and supplemented bronchial epithelial cell basal medium in the lower chamber.

Cell suspensions (150 µl, 1 x 10⁶ cells/ml) were added to the upper chamber and the Boyden Chambers were kept at 37°C for 6 h (H1299), 12 h (A549), 24 h (16HBE) or 48 h (16HBE). The chambers were washed with Dulbecco’s phosphate-buffered saline (PBS) buffer (PAOLA Laboratories GmbH) and cells were fixed with methanol (Sigma) for 10 min at –20°C. The cells were stained using Giemsa (Sigma) for 10 min at 4°C and rinsed with water. The cells on the upper side of the membrane were scraped off and the membranes were washed and dried overnight. After cutting out the membrane, cells were counted on four areas of the same size and the average number of cells per square millimeter was calculated. For each cell line, the migration time was determined experimentally in order to obtain a number of cells between 20 and 100 per membrane area. At least three independent experiments were performed for each condition.

**Three-dimensional tissue culture assay**

Substrates of collagen, chitosan and glycosaminoglycans were prepared as described previously (18). Three-dimensional cultures (TDC) were generated using a protocol derived from the one developed for skin tissue equivalents and were prepared by adding a suspension of 2 x 10⁵ human fibroblasts/cm² on top of the substrate in Dulbecco’s modified Eagle’s medium (DMEM with Glutamax, Life Technologies, Cergy Pontoise, France) supplemented with 10% fetal calf serum (HyClone, Logan, UT), antibiotics and 1 mM ascorbic acid 2-phosphate (Sigma). The medium was replaced every day. After 21 days, lung adenocarcinoma cells (A549 and H1299) were plated on the substrate colonized by fibroblasts at a concentration of 2 x 10⁵ cells/cm². The TDCs were kept in suspension in DMEM medium as described above with 1 mM ascorbic acid 2-phosphate for 7 days. Treatment with siRNA was started on the second day after seeding the cell. One TDC of each cell line was kept as control, one was treated with scramble RNA and one with siRNA CHRNA5. Experiments were performed in triplicate. TDCs were then brought to the air–liquid interface for an additional 14 days in medium, supplemented with 0.4 µg/ml hydrocortisone (Upjohn, St. Quentin en Yvelines, France), 5 µg/ml insulin (Lilly, Saint-Cloud, France), 8 mg/ml Bovine Serum Albumine (Sigma), 1 mM ascorbic acid 2-phosphate and antibiotics. TDCs were fixed with formaldehyde (4%) and embedded in paraffin. Five micrometer-thick sections were cut and stained with hematoxylin (Sigma).

**Immunofluorescence**

A549, TE1 and TE13 cells were cultured on glass cover slides. After treatment with siRNA as indicated, cells were rapidly washed with PBS and fixed with formaldehyde (4% in PBS) for 10 min at room temperature. After washing with PBS, cells were permeabilized with Triton X-100 (0.5% in PBS), blocked in Image-iTTM FX Signal enhancer solution (Invitrogen) and immunostained with primary antibodies anti-p120 (12H6) (monoclonal, 1:500; BD Transduction Laboratories, San Jose, CA) or Anti-ZO-1 (monoclonal, 1:100; Zymed, South San Francisco, CA). After washing, cells were incubated to secondary goat anti-mouse antibodies conjugated to Alexa Fluor® 488 (1:200 in PBS; Invitrogen) before DNA staining with ToPro3 (1:300 in PBS; Invitrogen). Stained slides were mounted using antifading medium (Invitrogen). For p63 staining, signal amplification was obtained using a horseradish peroxidase system. After permeabilization with Triton (0.5% in PBS), cells were treated with a H₂O₂ solution (5% in PBS; Sigma) prior to incubation on blocking buffer. The cells were then immunostained with the primary antibody anti-p63 (4A4, monoclonal, 1:500; GenTex, San Antonio, TX), washed again and incubated with peroxidases-conjugated secondary antibody, goat anti-mouse immunoglobulin, (GAM-HRP, 1:200 in PBS; Dako, Glostrup, Denmark) as secondary antibody. Cells were then exposed to tyramide-coupled fluorescent dye (Perkin Elmer Life Sciences, Boston, MA) before DNA staining with ToPro3 (1:300 in PBS). The fluorescent samples were examined with a Zeiss laser scanning confocal microscope LSM 510 on Axiovert 200M. The LSM 4.0 sp1 (Zeiss, Iena, Germany) software was used for image acquisition and analysis.

**Real-time quantitative polymerase chain reaction**

Total RNA was extracted using a Nucleospin RNA II kit (Machery-Nagel, Düren, Germany) and reverse transcription was performed using random primers (Promega) and SuperScript II (Invitrogen). RNA expression was analyzed by real-time quantitative polymerase chain reaction using Brilliant SYBR Green Master Mix (Qiagen) and 0.4 nM of primers as described previously (19). Expression levels were normalized to GAPDH. All samples were analyzed in duplicate and three independent real-time quantitative polymerase chain reactions were performed for each experiment.

**Protein extraction and western blotting**

Cells were lysed by adding lysis buffer [50 mM Tris–HCL, pH 7.4, 250 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% Nonidet P-40 (NP-40) containing protease inhibitors [2 µg/ml aprotinin, 500 µM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin and 2 mM dithiothreitol (Sigma)] and placed on ice for 30 min. Extracts were subsequently centrifuged at 13 000 rpm for 15 min at 4°C. Supernatants were collected. Protein concentration was determined using Bradford reagent (Bio-Rad; Bio-Rad Laboratories, Temeculah, CA). Thirty micrograms of protein extracts were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidinedifluoride (Amersham Biosciences, Piscataway, NJ) membranes by electroblotting. Membranes were blocked with 5% dry milk in PBS–NP-40 (0.5%) for 1 h at room temperature and incubated overnight with primary antibody diluted in 0.1% PBS–NP-40 containing 1% dry milk. The following antibodies were used: mouse monoclonal against p63 (A549, 1:500 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) and Ku80 (1:40 000 in PBS; Serotec, Raleigh, NC). Membranes were washed and incubated with peroxidases-conjugated goat anti-mouse [1:10 000 in PBS (Dako)]. Proteins were detected using the ECL™ detection kit (Amersham Biosciences).

**Calcium influx**

The cell line H1299 (1 x 10⁵ cells/ml) was seeded into glass chamber slides (Lab Tek, 154526; Thermo Scientific) and the cells were transfected with siRNA as described above. The medium was removed and the cells were incubated with a 1 µM solution of Fluoro-4 (Invitrogen) in Ringer solution (pH 7.2) with NaCl (250 mM), KCl (20 mM) and 1 at 37°C. Cells were washed once with Ringer solution and then left in Ringer solution for another hour before analysis. NACRhRs were stimulated by adding nicotine (final concentration 1 mM) directly into the Lab Tek chamber. Prior to nicotine treatment, cells were incubated for 30 min at 37°C with 2-β-bungarotoxin (final concentration 1 µM) or zα4-30ntoxin (final concentration 10 nM). The Fluoro-4 was excited with an Argon laser at minimum intensity using a ZEISS LSM 5 exciton microscope. Images were analyzed using the program Image J (http://rsbweb.nih.gov/ij/, Jan 2010).

**Flow cytometry**

DNA synthesis was analyzed by 5-bromo-2’-deoxyuridine (BrdU) incorporation. Cells were cultured on glass cover slides. After treatment with siRNA treatment, cells were incubated with BrdU (final concentration 1 µg/ml; BD Biosciences, San Jose, CA) for 90 min. Cells were detached, fixed with 70% ethanol and incubated with 20 µl anti-BrdU-FTTC (BD Biosciences) for 20 min. BrdU incorporation was measured using FACS Canto II as described by the suppliers (Becton Dickinson). Data were analyzed with BD FACSDIVA Software.

**Sequencing**

SNP rs16969968 was analyzed by PCR and direct sequencing. DNA was isolated from cell lines using Qiagen DNA Extraction Mini kit according to the manufacturer instructions (QiAamp DNA Mini Kit; Qiagen). PCR was performed with Taq Platinum (Invitrogen) and 0.4 µM (final concentration) primers 5’–GCTCTTGTGCAGCAGAATA-3’ (forward) and 5’–CATCGCTGT TATCTGCGCAAAA-3’ (reverse); Eurogentec, Seraing, Belgium). PCR included 2 min activation at 94°C, followed by 40 cycles of the program at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and 10 min extension at 72°C. Two independent PCRs were performed and negative controls were run in
parallel to monitor possible contamination. PCR products were visualized on 2% agarose gel stained with ethidium bromide prior to sequencing. Five microliters of PCR products were purified with the enzyme ExoSap-IT (USB, Cleveland, OH) for 15 min at 37°C and 15 min at 80°C. Sequencing was performed on ABI Prism 3100 Genetic Analyser (Applied Biosystems) with the primers used for the amplification.

Statistical analyses

For migration assays using Boyden Chambers, each experiment was repeated at least three times. Cell counts were averaged for these triplicate experiments and standard errors were calculated. Statistical analysis was conducted using a paired t-test assuming that experimental conditions correspond to population of equal variance. \( P < 0.05 \) (‘) was considered statistically significant. These statistical analyses were repeated using the ADAM software version 2.54 (German Cancer Research Center 2008). Pairwise non-parametric Wilcoxon test without Bonferroni adjustment for multiple testing was performed to assess differences between treatment and control. The significance level alpha was set to 0.005 (Figure 1A) or 0.05 (Figure 1B and C).

Results

Inhibition of CHRNA5 increases migration in non-transformed bronchial cell lines and in lung cancer cell lines

Previous studies have shown that \( \alpha_3 \beta_2 \), \( \alpha_5 \) nAChRs are involved in cell migration and in the modulation of calcium influx during wound healing of the respiratory epithelium (16). To determine the nature of this effect in non-transformed human bronchial epithelial cells, we treated 16HBE cells (SV40-immortalized bronchial cells) and NHBE cells (normal human primary bronchial epithelial cells) with nicotine, \( \alpha_3 \)-conotoxin MII [Ctx, a specific inhibitor of nAChR complexes containing \( \alpha_3 \beta_2 \)] has a nicotine-like effect.

To assess the role of the \( \alpha_5 \) nAChR subunit in cell migration, we silenced CHRNA5 expression using siRNA in two lung cancer cell lines with different CHRNA5 polymorphic status, A549 (rs16969968 GG, D398) and H1299 (rs16969968 AA, N398). Both cell lines were shown to express a wide range of nAChR subunits (Table I). The efficiency of gene silencing was assessed by reverse transcription polymerase chain reaction (see supplementary Figure S1, available at Carcinogenesis Online). In A549 and H1299 cells, expression of CHRNA5 was decreased by \( \sim 80\% \) compared with the siRNA control. Migration assays were performed in Boyden chambers with adapted migration times as detailed in Materials and Methods. Figure 1B shows the effects of nicotine, Ctx, Bgtx, siRNA to \( \alpha_5 \) or \( \alpha_7 \) nAChR, and combinations thereof, on the migration properties of A549 and H1299 cells. Treatment of either cell line with nicotine significantly increased invasiveness in both cell lines although the amplitude of this effect was greater in A549 (\( P < 0.05 \), Student’s t-test and \( P < 0.05 \), Wilcoxon test) than in H1299 cells (\( P < 0.05 \), Student’s t-test; \( P = 0.005 \) (Student’s t-test); ‘’\( P < 0.005 \) (pairwise non-parametric Wilcoxon test without Bonferroni adjustment).
non-significant, Wilcoxon test). Enhanced migration was also observed with Ctx but not with Bgtx, the latter even slightly decreasing migration in H1299 cells. Upon silencing CHRNA5, migration significantly increased in both cell lines. This effect was enhanced by addition of nicotine but was blocked by Bgtx, whereas Ctx had no significant effect. Finally, silencing CHRNA7 or of both CHRNA5 and 7 strongly reduced migration. Overall, these results suggest that A549 or H1299 cells responded to nicotine or nAChR antagonists in a manner similar to non-transformed bronchial cells. Blocking nAChR containing α5 with α-conotoxin MII or silencing CHRNA5 had a nicotine-like effect. Moreover, silencing CHRNA5 in A549 and H1299 cells increased the effects of nicotine. Taking into account that Ctx is a specific antagonist of receptor complexes containing α3 and β2 subunits which in bronchial cells are essentially represented by (α3β2)γ5 receptors, the lack of effect of Ctx in cells silenced for CHRNA5 suggests that inhibition of CHRNA5 expression suppresses signaling through these heteropentameric receptors. Thus, CHRNA5 appears to operate as a negative regulator of nicotine-induced cell migration. In contrast, inhibition of these effects in the presence of Bgtx or after silencing CHRNA7 indicates that the homopentameric α7 receptor operates as a positive regulator of cell migration.

To rule out the above effects may be affected by changes in cell proliferation patterns induced by various treatments, we silenced CHRNA5 and/or CHRNA7 in A549 or H1299 cells and measured the incorporation of BrdU as a marker of S-phase. Figure 1C shows that BrdU incorporation significantly decreased in cells with silenced CHRNA5 or CHRNA7 and that this effect was more marked in H1299 (60% decrease) than in A549 (20% decrease) (P < 0.005, Student’s t-test and Wilcoxon test).

To further evaluate the role of the nAChR α5 subunit on motility at the single cell level, we used videomicroscopy to quantify the two-dimensional movements of A549 and H1299 cells. Silencing of CHRNA5 in A549 cells increased the distance covered by the cells (P = 0.01, Kruskal–Wallis test) (supplementary Figure S2 is available at Carcinogenesis Online). Interestingly, in H1299, silencing of CHRNA5 did not influence their two-dimensional movements (data not shown), suggesting that migration (as measured in Boyden Chamber assays) and two-dimensional cell motility properties are distinctly affected by nicotine signaling.

Effects on CHRNA5 silencing on cell adhesion properties
To evaluate cell proliferation and migration in a TDC system, we seeded A549 or H1299 cells over a synthetic dermis equivalent made of polymerized glycosaminoglycans, chitosans and collagens, colonized with primary fibroblasts prior to cancer cell seeding (23). Comparison between cells transfected with either control or α5 siRNA showed that in both cell lines, the silencing of CHRNA5 increased the thickness of the reconstituted epithelium, blurred the delineation between the epithelium and the underlying dermis equivalent and increased the invasion of epithelial cells within the synthetic dermis layer (Figure 2A). These observations are consistent with an increased capacity of CHRNA5-silenced cells to invade the extracellular matrix. This increase in invasive capacity was observed with both A549 and H1299 cells but was particularly marked in the latter cell line (arrows on Figure 2A).

Increased cell motility and migration suggest that nicotinic receptor signaling may alter the expression of components of cell–cell and/or cell–matrix adhesion complexes. To assess the effects of the α5 receptor subunit on cell adhesion complexes, we analyzed the distribution of P120-catenin and ZO-1, which are components of adherens junctions and tight junctions, respectively, in A549 cells. Figure 2B shows that silencing of CHRNA5 reduced the expression of both adhesion molecules, with an almost complete disappearance of P120-catenin from cell–cell contacts and reduction and disorganization of ZO-1 staining. Overall, these results show that silencing CHRNA5 led to changes in the expression of cell adhesion patterns, compatible with loss of adhesion properties that may account for increased motility and invasiveness.

Effect of CHRNA5 silencing on calcium influx
The nicotine-like effects of CHRNA5 silencing on cell motility, invasiveness and expression of P120-catenin and ZO-1 are consistent with previous reports showing that nicotine induced proliferation, invasion and epithelial-to-mesenchymal transition in a variety of human cancer cell lines (24). Nicotinic receptors belong to the family of ligand-gated calcium channels, the activation of which mediates Ca2+ influx. Our results suggest that, similar to nicotine, silencing CHRNA5 may increase calcium influx and may also further enhance the influx triggered by nicotine. To test this hypothesis, we measured Ca2+ influx in H1299 cells using fluorescent probe Fluo-4. Silencing CHRNA5 considerably increased the effect of nicotine (1 mM) on Ca2+ influx, consistent with a negative effect of CHRNA5 on Ca2+ influx (Figure 3A and B). To further understand the different roles
Fig. 3a. Effects of nicotine and antagonists on calcium influx in cells with silenced CHRNA5. Calcium influx in H1299 (A–D), 16HBE (E–F) and NHBE (G–E) was detected using the fluorescent marker Fluo-4. Panels A, C, E and G: microphotographs of cells with artificial colors expressing Ca$^{2+}$ levels. Color ranges from blue (low amount of Ca$^{2+}$) to green, yellow and red (high amount of Ca$^{2+}$). Panels B, D, F and G: quantitative analysis of Ca$^{2+}$ influx in H1299 was performed using the Image J analysis program (http://rsbweb.nih.gov/ij/, Jan 2010). Nicotine was added at $t = 0$ sec (final concentration of nicotine: 1 mM) to cells transfected with siRNA CHRNA5 (A and B) or treated with α-conotoxin MII (Ctx, 10 nM) or α-bungarotoxin (Bgtx, 1 μM) (C–G).
of CHRNA5 and CHRNA7, we treated H1299 cells with either Ctx or Bgtx and measured the Ca\(^{2+}\) influx induced by nicotine. As shown in Figure 3C and D, Ctx increased calcium influx by >10-fold, whereas Bgtx almost completely inhibited calcium influx. Similar effects, although of lower amplitude, were observed in both non-transformed primary NHBE cells and immortalized 16HBE cells (Figure 3E–H). Overall, these results suggest that the expression of CHRNA5 contributes to the formation of nAChR subunits that exert an inhibitory effect on calcium influx mediated by nicotine through the homopentameric α7 nAChR, as well as on the subsequent signaling pathways controlling cell motility and migration.

**Downregulation of CHRNA5 decreases the expression of p63**

Recent studies have shown that p63, a protein with structural similarities with the tumor suppressor protein p53, is a critical regulator of epithelial cell differentiation, adhesion and survival, including bronchial cells (25–27). In bronchial cells as in most epithelial cells, p63 is expressed as multiple isoforms including in particular TA and DeltaN isoforms, the latter being devoid of the N-terminal, transactivation domain of the TA isoforms. In squamous cell lung carcinoma, TP63, the gene encoding p63, is often amplified and systematically overexpressed, leading to the accumulation of the DeltaNp63 isoform, which is constitutively expressed only in the basal cells of the normal epithelium (28). To determine if silencing of specific CHRNA subunits may affect p63 expression, we used two squamous cell carcinoma cell lines derived from esophageal cancers, TE1 and TE13. We selected these cells for this experiment on the basis of their previous characterization as expressing either low (TE1) or high (TE13) levels of DeltaNp63α (29,30). These two cell lines express multiple nAChR subunits (Table I). In both cell lines, CHRNA3, 5 or 7 were silenced and the efficiency of gene silencing was assessed by reverse transcription polymerase chain reaction (supplementary Figure 1C is available at Carcinogenesis Online). Figure 4A shows that silencing of either CHRNA3 or CHRNA5 induced a decrease in DeltaNp63 messenger RNA levels as measured by reverse transcription polymerase chain reaction, this effect being significant for CHRNA3 silencing in TE13 and for CHRNA5 silencing in both cell lines. In contrast, no significant reduction was observed with siRNA to CHRNA7. Figure 4B shows the reduction in nuclear levels of DeltaNp63 protein after silencing either CHRNA3 or CHRNA5, whereas silencing CHRNA7 had no effect, and even increased DeltaNp63 expression in TE13 cells. Western blot analysis of DeltaNp63α protein levels (Figure 4C) showed a strong reduction of protein levels in both cells silenced for CHRNA5. With siRNA to CHRNA3, DeltaNp63 became undetectable in TE1 and was reduced in TE13. In contrast, after CHRNA7 silencing, p63 protein levels were essentially unaffected and even increased in comparison with Ku80 levels in both cell lines. These results indicate that α7 subunits, on the one hand, and α3 or α5 subunits, on the other, have opposite effects on the regulation of DeltaNp63α.

**Discussion**

The strong association of loci encoding nAChR on 15q25.1 with risk for lung cancer or chronic obstructive pulmonary disease has been interpreted as the result of an increased propensity to smoking due to nicotine addiction in subjects carrying distinct nAChR variants [reviewed in ref. (31)]. However, the association is detectable in never smokers although there is controversy between different studies in this respect. A detailed analysis of CHRNA5 SNP rs16969968 in a total of 17 300 subjects from five lung cancer studies and four upper aerodigestive tract cancer studies has identified that subjects with the homozygous minor genotype (AA) smoked on average 1.2 cigarettes per...
day more than subjects with a GG genotype ($P < 0.001$). Moreover, the association between the variant and lung cancer risk was virtually unchanged after adjusting for smoking (32).

There is biological evidence for a role for nicotine and nAChRs in regulating the proliferation, differentiation and migration of bronchial epithelial cells. Studies in normal bronchial cells have demonstrated that ($\alpha3\beta2\gamma2$) receptors were expressed during wound healing in the normal mucosa and that this expression was particularly high at the edges of the wounds (16). In contrast, the homopentameric $\alpha7$ receptors, which are not encoded by genes located in a lung cancer susceptibility region, were involved in the control of the proliferation of human airway epithelial basal cells (17).

In this study, we show that CHRNA5 plays a significant role in modulating adhesion and motility in both transformed and non-transformed bronchial cells, as well as in regulating the expression of p63, a potential oncogene in squamous cell carcinoma (25,28). Using $\alpha$-conotoxin MII as a specific antagonist of heteropentameric receptor complexes (containing $\alpha5$) or silencing of CHRNA5 expression by RNA interference, we show that inhibition of receptors containing the $\alpha5$ subunit induces a nicotine-type effect and increases the effects of nicotine on cell migration in a three-dimensional invasion assay (Boyden Chamber). In contrast, silencing CHRNA7 or inhibition of $\alpha7$ with the antagonist $\alpha$-bungarotoxin decreased cell migration and counteracted the effects of inhibition of $\alpha5$. Similar effects (although with different amplitudes) were observed in transformed and non-transformed cells, independently of the polymorphic status of rs16969968.

The effects of nicotine, antagonists and RNA silencing on cell migration were compatible with the effects on calcium influx. In agreement with the nicotine-like effect of $\alpha5$ inhibition, silencing of CHRNA5 or treatment with $\alpha$-conotoxin MII increased the wave of $Ca^{2+}$ induced by nicotine. Conversely, treatment with $\alpha$-bungarotoxin inhibited $Ca^{2+}$ influx almost completely. Again, similar effects were observed in transformed and non-transformed cells. Overall, these results are compatible with the hypothesis that the $\alpha5$ subunit is a component of receptors operating as negative regulators of calcium influx through the homopentameric $\alpha7$ receptor.

The effects of $\alpha5$ inhibition on cell motility and migration were correlated with changes in expression of cell–cell adhesion molecules, with a reduction of components of tight junctions (ZO-1) and of adherens junctions (P120) in A549 cells silenced for CHRNA5. Diminished expression of adhesion molecules and increased invasiveness are two properties of epithelial cells undergoing epithelial-to-mesenchyme transition (33). Furthermore, silencing either CHRNA3 or CHRNA5 decreased the expression and nuclear accumulation of DeltaNp63, an isoform of the p63 protein that is constitutively expressed in basal and proliferating bronchial epithelial cells (27). Again, this effect was not observed upon silencing CHRNA7, which appeared to increase DeltaNp63 expression. These results support that nAChR $\alpha5$ subunit is part of a receptor operating as repressor of adhesion and migration in bronchial cells. Of note, silencing CHRNA3 in bronchial cancer cell lines H1299 and H1650 had similar effects on $Ca^{2+}$ influx as silencing CHRNA5 (34), suggesting that this repressor effect is a property of the ($\alpha3\beta2\gamma2$) receptor complex rather than a specific property of the $\alpha5$ subunit. Recent studies have shown that the expression of several nAChR subunits is deregulated in primary lung cancer tissues (19,34,35). Moreover, CHRNA5, but not CHRNA3, is often hypermethylated and downregulated in cancer tissues, and a 30-fold upregulation of CHRNA5 expression has been observed in lung cancers as compared with the normal lung. Restoration of CHRNA3 by ectopic expression in the H1975 lung cancer cell line induced apoptotic cell death (34). These observations indicate that the expression and functions of ($\alpha3\beta2\gamma2$) receptor complexes are altered in primary lung cancers.

Our observations do not explain how polymorphisms of CHRNA5 may affect lung cancer susceptibility. The non-silent polymorphism rs16969968 falls within a conserved region of the second intracytoplasmic loop of the protein. The frequent allele (G) encodes an aspartic acid (D) at position 398, whereas the rare A allele (carrying the increased risk of lung cancer) encodes an asparagine (N). It is possible that high-risk 398N protein variant exerts a less potent negative effect on nicotine signaling than the low-risk variant (398D) protein variant, thus making bronchial cells more susceptible to the proliferating or motility-promoting effects of nicotine mediated through $\alpha7$ homopentameric receptor complexes. 

**Fig. 5.** Role ($\alpha3\beta2\gamma2$) nAChR complexes in regulating nicotine-induced calcium flux: a model. This diagram illustrates the negative role of ($\alpha3\beta2\gamma2$) nAChR complexes in the regulation of nicotine signaling through homopentameric $\alpha7$ receptors and its impact on cell adhesion, migration/invasiveness and p63 expression. This model suggests that the susceptibility associated with CHRNA3 variants may be due to differences in their capacity to exert a negative regulation on signaling through $\alpha7$ receptors. According to this hypothesis, the high-risk $\alpha3$ protein variant (398N) may exert a less potent negative regulatory effect on nicotine signaling than the low-risk variant (398D) protein variant, thus making bronchial cells more susceptible to the proliferating or motility-promoting effects of nicotine mediated through $\alpha7$ homopentameric receptor complexes.
the activity of these receptors, the mucosa may undergo rapid repair and recovery or in contrast undergo modifications to form hyperplastic or metaplastic areas that are precursors of cancer lesions. Polymorphisms in CHRNA5 genes may thus influence cancer risk by modulating the dynamics of the normal bronchial epithelium under stress conditions (31). Another, non-exclusive mechanism may consist in enhanced invasiveness and metastatic capacity. Moreover, the effects of the CHRNA5 polymorphism at the mucosal level may be compounded by neuronal effects on addiction to nicotine, increasing tobacco consumption and exposure to tobacco carcinogens (38). Together, these effects may account for the role of CHRNA5 variations as risk factors for a broad range of lung cancer aetiologies and histologies, as well as in non-cancer lung diseases that involve extensive tissue repair and remodeling, such as chronic obstructive pulmonary disease (6).

Supplementary material

Supplementary Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/.

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References


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