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ASCL1 Regulates Expression of the CHRNA5/A3/B4 Lung Cancer Susceptibility Locus

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Abstract

Tobacco contains a variety of carcinogens as well as the addictive compound nicotine. Nicotine addiction begins with the binding of nicotine to its cognate receptor, the nicotinic acetylcholine receptor (nAChR). Genome-wide association studies have implicated the nAChR gene cluster, CHRNA5/A3/B4, in nicotine addiction and lung cancer susceptibility. To further delineate the role of this gene cluster in lung cancer, we examined the expression levels of these three genes as well as other members of the nAChR gene family in lung cancer cell lines and patient samples using quantitative reverse transcription polymerase chain reaction. Over-expression of the clustered nAChR genes was observed in small cell lung carcinoma (SCLC), an aggressive form of lung cancer highly associated with cigarette smoking. The over-expression of the genomically clustered genes in SCLC suggests their coordinate regulation. *In silico* analysis of the promoter regions of these genes revealed putative binding sites in all three promoters for achaete-scute complex homolog-1 (ASCL1), a transcription factor implicated in the pathogenesis of SCLC, raising the possibility that this factor may regulate expression of the clustered nAChR genes. Consistent with this idea, knockdown of ASCL1 in SCLC, but not NSCLC, led to a significant decrease in expression of the α3 and β4 genes, without having an effect on any other highly expressed nAChR gene. Our data indicate a specific role for ASCL1 in regulating expression of the CHRNA3/A5/B4 lung cancer susceptibility locus. This regulation may contribute to the predicted role ASCL1 plays in SCLC tumorigenesis.

Keywords

Nicotinic Acetylcholine Receptors; CHRNA5/A3/B4 locus; Achaete-Scute Complex Homolog-1; Lung Cancer; Small Cell Lung Carcinoma

Introduction

Lung cancer is the leading cause of cancer-related mortality across the globe (1). Cigarette smoking and second-hand smoke are the major etiologic factors associated with lung cancer, accounting for nearly 90% of all lung cancer deaths. Given that 25% of adults smoke, a considerable number of people are presently at risk for the disease.

Lung cancer is classified into two main histological types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The latter can be further divided into large cell carcinoma, adenocarcinoma and squamous cell carcinoma. SCLC, a neuroendocrine tumor, is the most aggressive among the various types of lung cancer and has the poorest prognosis, with

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a 5-year survival rate of 15% (2). This can reach as low as 2% for patients diagnosed with late-stage disease. Though most patients respond to initial cycles of chemotherapy, they eventually become chemoresistant.

Nearly all SCLC patients (>95%) have a history of cigarette smoking (2). This strong etiologic link is not surprising given the fact that tobacco contains at least 55 carcinogens, the most potent of which are nicotine-derived nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (3). Increasing evidence also suggest that nicotine itself may directly contribute to carcinogenesis by inducing cell proliferation, transformation, apoptotic inhibition, and angiogenesis (4–6).

Nicotine and NNK are both exogenous ligands of nicotinic acetylcholine receptors (nAChRs) (7). nAChRs are transmembrane ligand-gated ion channels that have been extensively studied with respect to their role in fundamental physiological processes such as muscle contraction, attention, arousal, anxiety and learning and memory (8). They are key players in the nicotine reward pathway, making them attractive drug targets for smoking cessation therapies (9–12).

nAChRs have traditionally been referred to as either “muscle” or “neuronal” based on their expression patterns and subunit composition. Muscle nAChRs are made up of α1 subunits combined with β1, γ, δ, or ε subunits. Here we focus on neuronal nAChRs, pentameric structures made up of homomeric or heteromeric combinations of α and β subunits that include α2 – α10 and β2 – β4 (8). The precise combination of subunits determines the pharmacological and biophysical properties of the receptor (13,14). While the complete repertoire of native nAChRs has not been fully elucidated, it is clear that a staggering diversity of receptor subtypes and functions may exist (14).

The neuronal nAChRs have also been found in non-neuronal tissues (15–17). In particular, they are expressed in normal as well as lung cancer cells (18,19). The two most well-characterized nAChRs in this system are the homomeric α7 and the heteromeric α4β2 subtypes (6). Recently, however, a series of genome-wide association studies pointed to a possible role for the nAChR α3β4α5 subytpe in the etiology of lung cancer (20–23). These studies identified a lung cancer susceptibility locus in the long arm of chromosome 15 (15q24/15q25.1), a genomic region containing the genes encoding the α5, α3, and β4 subunits (CHRNA5/A3/B4). Single nucleotide polymorphisms found in the gene cluster were also found in independent studies to be associated with nicotine addiction (24–30). It is not yet clear how variants in this locus may modulate the function of mature nAChRs but this body of data does prompt further investigation on the role of these specific nAChR subunits in lung cancer.

To address this gap in knowledge, we first examined the expression profile of these genes as well as all other neuronal nAChR genes in lung cancer cell lines and patient samples. Here we describe the over-expression of the clustered nAChR genes in SCLC. Furthermore, we identified a transcription factor, ASCL1, that regulates the CHRNA3/A5/B4 gene cluster in this tumor type. ASCL1 is a basic helix-loop-helix transcription factor that binds to DNA recognition motifs known as E-boxes (31). It is over-expressed in SCLC and other neuroendocrine tumors. ASCL1 expression appears to be important for SCLC tumor initiation while its knockdown causes cell cycle arrest and apoptosis (32,33). In addition, transgenic mice that constitutively express ASCL1 and the SV40 Large T antigen develop aggressive lung tumors with neuroendocrine features (34). Over-expression of ASCL1 in SCLC may thus lead to corresponding over-expression of the clustered nAChR genes, providing a mechanism by which nicotine’s effects may be potentiated in SCLC, contributing to its increased tumorigenicity.

Results

Over-expression of Nicotinic Acetylcholine Receptor Genes in Lung Cancer

Quantitative RT-PCR was performed to compare mRNA expression of the neuronal nAChR gene family in normal and lung cancer cell lines. At least four lines derived from each of the major lung cancer types (SCLC, large cell lung carcinoma, adenocarcinoma and squamous cell carcinoma) were used in this analysis (see Materials and Methods for details). As shown in Figure 1, two of the clustered nAChR genes, those encoding the α3 and β4 subunits, were significantly over-expressed in SCLC lines compared to normal lung cell lines. In contrast, expression of the α5 subunit gene was high in all the cell lines studied including normal lung cell lines, while no significant differences in α5 expression were seen in any of the cell lines. Conversely, the α3 and β4 subunits were lowly expressed in large cell, adenocarcinoma and squamous cell carcinoma lines, similar to that in normal lung cell lines. With respect to the non-clustered nAChR subunit genes, the α4, α7, α10, and β2 genes were also significantly over-expressed in SCLC compared to normal lung cell lines (Fig. 2). In addition, the α7 gene was significantly over-expressed in large cell carcinoma cell lines (Fig. 2D).

Using a more physiologically relevant approach, we analyzed mRNA expression of the same set of genes in normal and lung cancer samples. The tumor samples were from patients with SCLC, large cell lung carcinoma, adenocarcinoma and squamous cell carcinoma (Table 1). Expression of all of the nAChR subunit genes was low in normal lung tissue. In comparison, all three of the clustered nAChR genes were significantly over-expressed in SCLC (Fig. 3). The α5 subunit gene was also significantly over-expressed in all NSCLC samples (Fig. 3B) while the β4 subunit gene was significantly over-expressed in adenocarcinoma and squamous cell carcinomas (Fig. 3C).

In SCLC samples, the nAChR α9 and β2 subunit genes were significantly over-expressed compared to normal lung tissue (Fig. 4E and G). With respect to non-small cell lung cancer, the β2 subunit gene was significantly over-expressed in adenocarcinoma and squamous cell carcinoma (Fig. 4G). In contrast, nAChR α2 subunit gene expression was significantly lower in all lung cancer tissues compared to normal lung tissue (Fig. 4A).

E-boxes are Present in the Promoters of the Clustered Nicotinic Receptor Genes

The high expression of the α3, α5, and β4 genes in SCLC as well as their genomic clustering suggests that they may be coordinately regulated (35). As an initial approach to identifying regulatory factors of this gene locus, *in silico* tools were used to analyze the promoter region of each gene for potential transcription factor binding sites. A number of putative binding sites for basic helix-loop-helix transcription factors were identified (Fig. 5). These sites are referred to as E-boxes and have the core sequence 5'-CANNTG-3'. The α3 gene promoter contains two E-boxes with the sequences CAGGTG and CACCTG. The α5 gene promoter contains four E-boxes with the sequences CAAATG, CAGCTG, CACCTG, and CACATG while the β4 gene promoter contains five E-boxes with the sequences CATTTG, CACATG, CAGCTG, and two CAGGTGs. With the exception of one E-box in the β4 promoter, all E-boxes are located upstream of reported major transcription initiation sites (36–38).

ASCL1 Differentially Regulates Expression of the Clustered Nicotinic Receptor Genes

Although there is a large family of basic helix-loop-helix transcription factors, we focused on ASCL1 because of its critical role in SCLC, as described above. To determine whether ASCL1 regulates expression of nicotinic receptor genes, knockdown experiments were done in SCLC cell lines using small interfering RNAs (siRNAs) against ASCL1. To control for off-target effects, three distinct siRNAs were used. The most potent siRNA, s1656, reduced ASCL1 mRNA expression by approximately 87% leading to an 89% decrease in α3 gene expression,

a 45% decrease in α5 gene expression and a 78% decrease in β4 gene expression (Fig. 6A, left). The second siRNA, s1657, reduced ASCL1 mRNA expression by 64% leading to a 77% decrease in α3 gene expression, an 18% decrease in α5 gene expression and a 66% decrease in β4 gene expression (Fig. 6A, middle). The third siRNA, s1658, reduced ASCL1 mRNA expression by 65% leading to a 78% decrease in α3 gene expression, a 17% decrease in α5 gene expression and a 41% decrease in β4 gene expression (Fig. 6A, right). Decreases in α5 expression were not found to be statistically significant. In addition, ASCL1 knockdown did not significantly affect the expression of the genes encoding the α7 and β2 subunit genes, two other nAChR subunits implicated in lung cancer, indicating specificity of α3 and β4 subunit gene regulation by ASCL1. ASCL1 knockdown also did not affect the expression of the housekeeping gene, GAPDH (data not shown). Furthermore, knockdown of ASCL1 in a non-small cell lung carcinoma cell line, A549, did not reduce expression of the α3, α5, and β4 subunit genes (Fig. 6B). Expression of the β2 subunit gene, however, appears to increase in this cell line upon ASCL1 knockdown. Western blot analysis confirmed that ASCL1 knockdown was achieved at the protein level (Fig. 6C).

Discussion

Our observation that the nAChR α3, α5 and β4 subunit genes are over-expressed in SCLC is particularly intriguing in light of the recent genome wide association studies implicating the CHNRA5/A3/B4 gene locus in lung cancer susceptibility (20–23). Over-expression of the clustered nAChR genes in lung cancer cells supports the notion that these genes play a role independent of the nicotine addiction pathway. Extrapolating on data gained from work in the nervous system and our own observations, the possible nAChR subtypes that can form in SCLC include α3β2, α3β4, α3β4α5, and α3β2β4α5 (39). These subtypes are believed to be involved in ganglionic neurotransmission in the peripheral nervous system (40). A thorough investigation of functional nAChR subtypes in lung cancer has yet to be done but there is evidence that specific subtypes mediate distinct processes. For example, α3-containing nAChR subtypes have been implicated in nicotine-mediated activation of the Akt pathway (41) whereas the α7 subtype is thought to mediate nicotine-induced angiogenesis and NNK-induced apoptotic inhibition (4,41). α7 nAChRs also have high calcium permeability and binding of NNK results in calcium influx, which triggers signaling pathways that result in cell proliferation, increased cell migration, apoptotic inhibition, and angiogenesis (6). These two examples indicate the need to identify all of the precise nAChR subtypes in lung cancer cells as this may be important for design of targeted therapeutics given the unique pharmacological and functional properties of each nAChR subtype.

As nAChRs are the cognate receptors for nicotine and NNK, their activation is likely the first step in signal transduction cascades involving these ligands. Persistent activation of cancer-promoting pathways has been shown to result from nicotine and NNK exposure and may facilitate SCLC development (42,43). While these pathways remain to be completely elucidated, they appear to involve the mitogen activated kinases ERK1 and ERK2, protein kinase C (PKC), the serine/threonine kinase RAF1 and the transcription factors FOS, JUN and MYC (6). In addition, exposure to nicotine has also been shown to reduce the efficacy of anti-cancer agents by inhibiting apoptosis (44). Pharmacological approaches suggest that these effects are mediated at least in part by homomeric α7 nAChRs (6) but the role of other nAChR subtypes cannot be ruled out due to the lack of specificity of currently available pharmacological agents.

That nAChRs may function in SCLC is not totally unexpected given their important role in the nervous system. SCLC is believed to develop from pulmonary neuroendocrine cells. As the name suggests, these cells share properties with neurons such as the expression of ion channels and neuropeptides and have been referred to as paraneurons (45).

From a regulatory standpoint, the over-expression of the clustered nAChR genes also yields some interesting insights. Several laboratories have previously identified regulatory features shared by these genes (36,46–53). Based on these studies, it is believed that expression of the clustered nAChR genes results from interactions between ubiquitously expressed and cell-type-specific transcription factors with cis-acting regulatory elements located within or near the cluster. To date, only one cell-type-specific factor, Sox10, has been identified and shown to regulate nAChR gene expression (54,55). Sox10 activates the promoters of the clustered genes in neuronal cell lines but not in non-neuronal cells. However, we have observed that Sox10 is not expressed in any of the lung cancer cell lines we used in this study (data not shown). This suggests that other transcription factors must be involved in the expression of nAChR genes in lung cancer. As mentioned above, the transcription factor ASCL1 is an interesting candidate given its role in SCLC (31–34). ASCL1 is also known to activate neuroendocrine differentiation markers while suppressing putative tumor suppressor genes (56). In addition, ASCL1 is required for the proper development of peripheral sympathoadrenal tissues, the same tissues where the clustered nAChR genes are abundantly expressed (57).

The knockdown experiments presented here indicate that ASCL1 robustly regulates the expression of the α3 and β4 genes while α5 gene expression was, at most, modestly affected. These regulatory differences are likely due to the fact that each gene has its own promoter. Hence, although the three genes share common regulatory elements, each gene may have additional mechanisms that allow fine-tuning of its specific expression. Moreover, the α5 gene is transcribed in the opposite direction as the α3 and β4 genes raising the possibility that transcription factors that bind to the α3 and β4 promoters may be differentially utilized by the α5 promoter and vice versa. Nevertheless, the effect of ASCL1 on nAChR subunit gene expression in SCLC appears to be specific for the clustered subunit genes, as expression of the α7 and β2 genes was not affected by ASCL1 knockdown. In contrast, ASCL1 knockdown does not reduce the expression of the clustered subunit genes in NSCLC whereas it increases the expression of the β2 gene, suggesting cell-type specificity of ASCL1 regulation.

Control of nAChR gene expression by ASCL1 may provide a mechanism for the role of nicotine in lung cancer. Nicotine has been shown to induce cellular processes that may lead to the development of cancer including activation of cell proliferation and survival pathways (6). Acetylcholine, the endogenous ligand for nAChRs, is also thought to act as an autocrine growth factor in lung cancer cells (58). Over-expression of their cognate receptors via transcriptional control by ASCL1 may thereby potentiate the effects of these ligands, providing a mechanism by which cigarette smoking can promote the growth and aggressiveness of SCLC.

Materials and methods

Cell lines

Cell lines were obtained from the American Type Culture Collection (ATCC) and passaged immediately upon receipt. SCLC cell lines were DMS-53, DMS-114, NCI-H69, NCI-H82, NCI-128, NCI-146, NCI-H209 and NCI-446. NSCLC cell lines were the large cell lung carcinoma cell lines NCI-H460, NCI-H661, NCI-1581 and NCI-H1915; the lung adenocarcinoma cell lines A549, NCI-H838, NCI-H1395, NCI-H1734 and NCI-H1793; and the squamous cell lung carcinoma cell lines NCI-H520, NCI-H1869, NCI-H2170, SK-MES-1 and SW-900. Normal lung cell lines were BEAS-2B, HBE4-E6/E7, LL-24 and WI-38. Cell lines were maintained in ATCC-recommended media at 37°C and 8% CO_2.

Patient samples

Tissue samples were obtained from the UMass Cancer Center Tissue Bank and the Cooperative Human Tissue Network. Approval from the University of Massachusetts Medical School

Institutional Review Board was obtained prior to sample collection. To date, a total of 123 cancer and normal lung tissues have been collected consisting of 53 normal, 7 SCLC and 63 NSCLC tissues including 19 adenocarcinomas, 32 squamous cell lung carcinomas and 12 large cell lung carcinomas. Samples were either snap-frozen surgically resected tissues or fresh pleural effusions. Available normal attached tissues were used as controls.

Quantitative RT-PCR

Total RNA was isolated from the cell lines and patient tissues using a RiboPure Kit (Ambion). cDNAs were generated using a RETROscript Kit (Ambion). Quantitative RT-PCR was performed using an ABI 7500 Real-Time System and ABI TaqMan assays for nAChR α2-α7, α9-α10 and β2-β4. α8 gene expression was not analyzed because its expression has only been observed in avian species. Samples containing no reverse transcriptase were used as negative controls. To confirm specificity, representative samples were analyzed in 2% agarose gels (data not shown). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene β2-microglobulin was used as the endogenous control.

ASCL1 knockdown

Knockdown of ASCL1 expression was performed in a SCLC cell line, DMS-53 and a NSCLC cell line, A549. To control for off-target effects, three different siRNAs against ASCL1 were used namely s1656, s1657, and s1658 (ABI). Transient transfections were performed using Lipofectamine™2000 (Invitrogen). Knockdown levels were determined using quantitative RT-PCR. A negative control siRNA (ABI) that does not target any known human, mouse, or rat gene was used to normalize gene expression. Untransfected samples were also analyzed for baseline gene expression. Corresponding changes in nAChR α3, α5, and β4 gene expression was measured using quantitative RT-PCR with β2 microglobulin as endogenous control. To determine specificity, gene expression of β2 was also measured. GAPDH levels were measured as a negative control. Samples were analyzed in triplicate and at least two independent experiments were done for each siRNA.

Immunoblotting

Western blot analysis was performed using standard procedures to determine ASCL1 knockdown levels. Briefly, 50 μg of DMS-53 lysates were loaded into 10% SDS-PAGE gels then transferred to nitrocellulose membranes. Membranes were incubated with ASCL1 and β2-microglobulin antibodies followed by goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology). Bands were visualized using a SuperSignal West Dura Extended Duration Substrate chemiluminescence kit (Pierce) and a VersaDoc Imaging System (Bio-Rad).

Statistical analysis

The mean relative expression values of each gene in the different samples were calculated and subjected to statistical analysis using the GraphPad Prism software. One-way analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison post-test.

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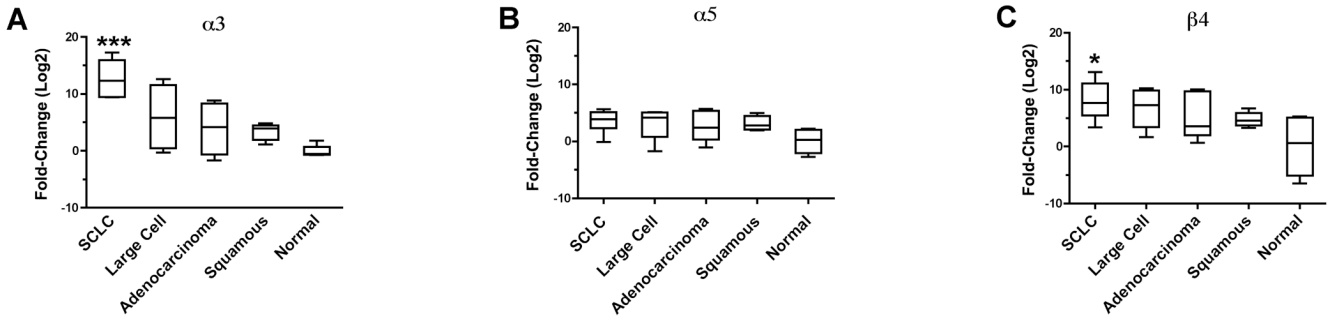

FIGURE 1.

Differential expression of the nAChR α3 (**A**), α5 (**B**), and β4 (**C**) genes across different cell lines. Quantitative RT-PCR was performed to compare expression levels between lung cancer cell lines and normal lung cell lines. Samples were analyzed in triplicate. The line within each box represents the median fold-change relative to normal. The upper and lower edges of each box represent the 75th and 25th percentile while the upper and lower bars represent the maximum and minimum values, respectively. Asterisks indicate statistical significance compared to normal lung cell lines (* = p value < 0.05, *** = p value < 0.001).

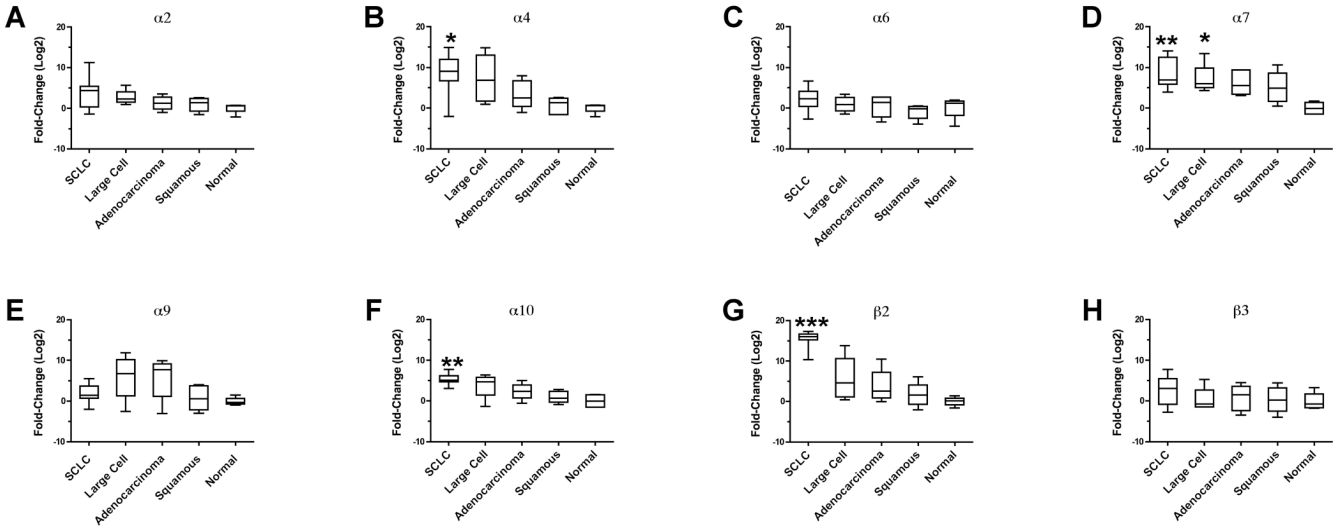

FIGURE 2.

Differential expression of the nAChR α2 (**A**), α4 (**B**), α6 (**C**), α7 (**D**), α9 (**E**), α10 (**F**), β2 (**G**) and β3 (**H**) genes across different lung cell lines. Quantitative RT-PCR was performed as described in Materials and Methods. Samples were analyzed in triplicate. The line within each box represents the median fold-change relative to normal. The upper and lower edges of each box represent the 75th and 25th percentile while the upper and lower bars represent the maximum and minimum values, respectively. Asterisks indicate statistical significance compared to normal lung cell lines (* = p value < 0.05, ** = p value < 0.01 *** = p value < 0.001).

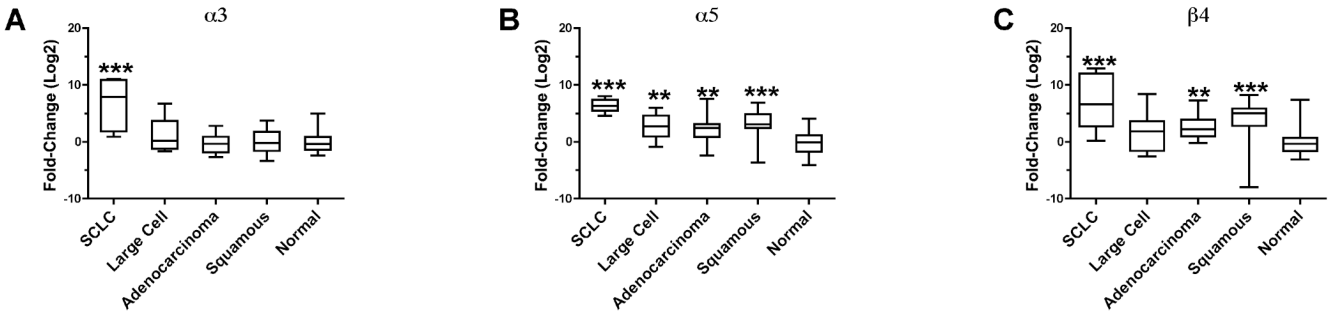

FIGURE 3.

Over-expression of the nAChR α3 (**A**), α5 (**B**), and β4 (**C**) genes in lung cancer patient samples. Quantitative RT-PCR was performed to compare expression levels between lung cancer patient samples and normal lung tissue. Samples were analyzed in triplicate. The line within each box represents the median fold-change relative to normal. The upper and lower edges of each box represent the 75th and 25th percentile while the upper and lower bars represent the maximum and minimum values, respectively. Asterisks indicate statistical significance compared to normal lung tissue (** = p value < 0.01 *** = p value < 0.001).

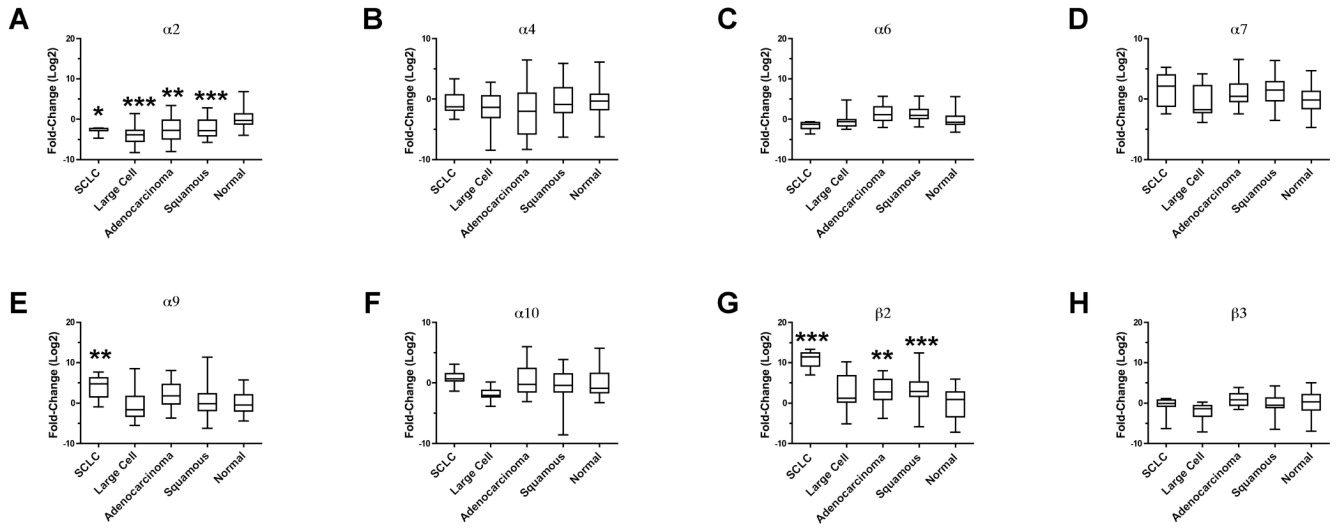

FIGURE 4.

Differential expression of the nAChR α2 (**A**), α4 (**B**), α6 (**C**), α7 (**D**), α9 (**E**), α10 (**F**), β2 (**G**) and β3 (**H**) genes across different lung cancer patient samples. Quantitative RT-PCR was performed as described in Materials and Methods. Samples were analyzed in triplicate. The line within each box represents the median fold-change relative to normal. The upper and lower edges of each box represent the 75th and 25th percentile while the upper and lower bars represent the maximum and minimum values, respectively. Asterisks indicate statistical significance compared to normal lung cell lines (* = p value < 0.05, ** = p value < 0.01 *** = p value < 0.001).

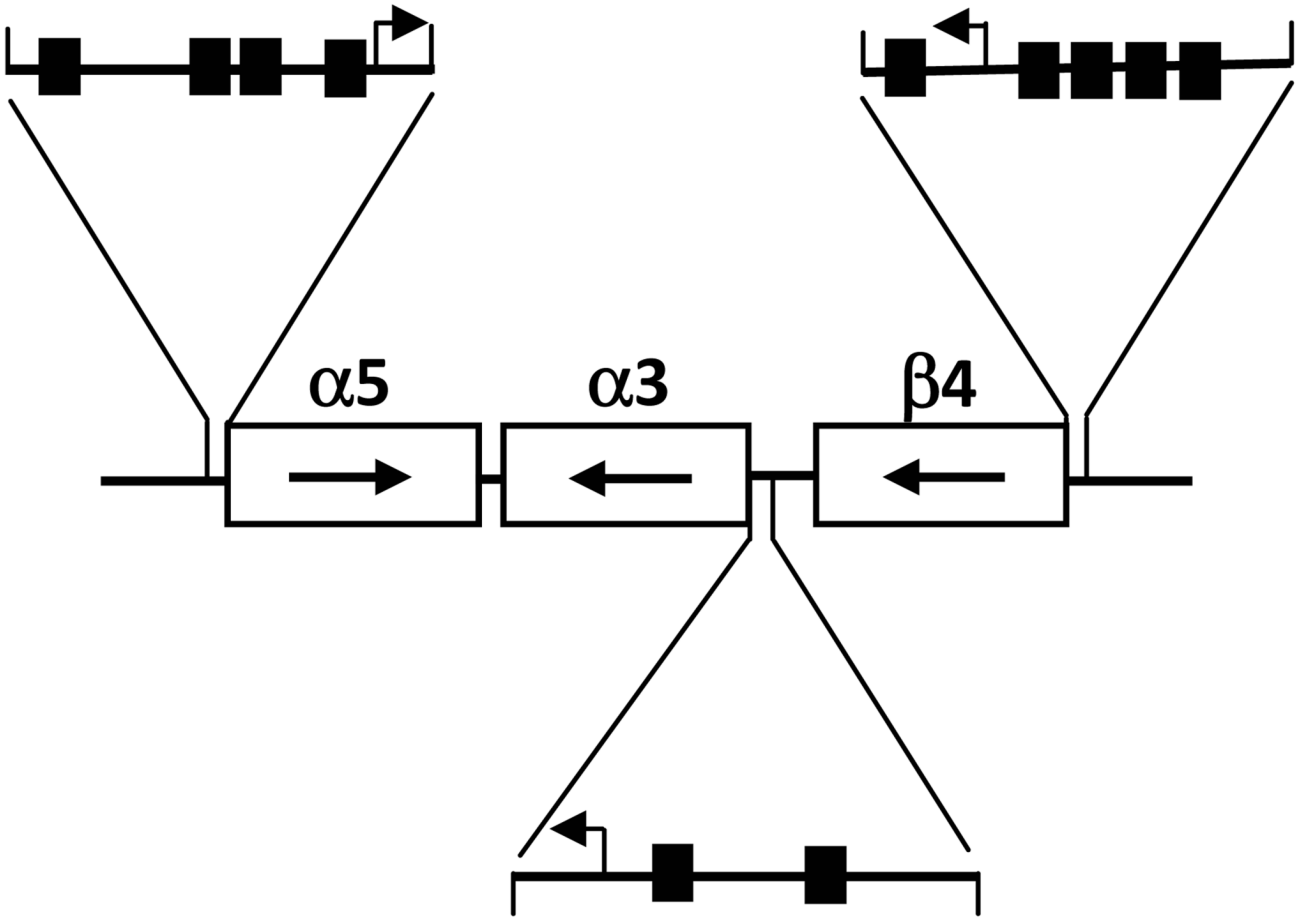

FIGURE 5.

The CHRNA5/A3/B4 promoter regions contain putative ASCL1 binding sites. The α5, α3, and β4 genes are clustered in the genome (white boxes). Straight arrows indicate directions of transcription. Bent arrows indicate major transcription initiation sites (36–38). Potential ASCL1 binding sites (E-boxes) in the promoter regions of these genes are indicated (black boxes).

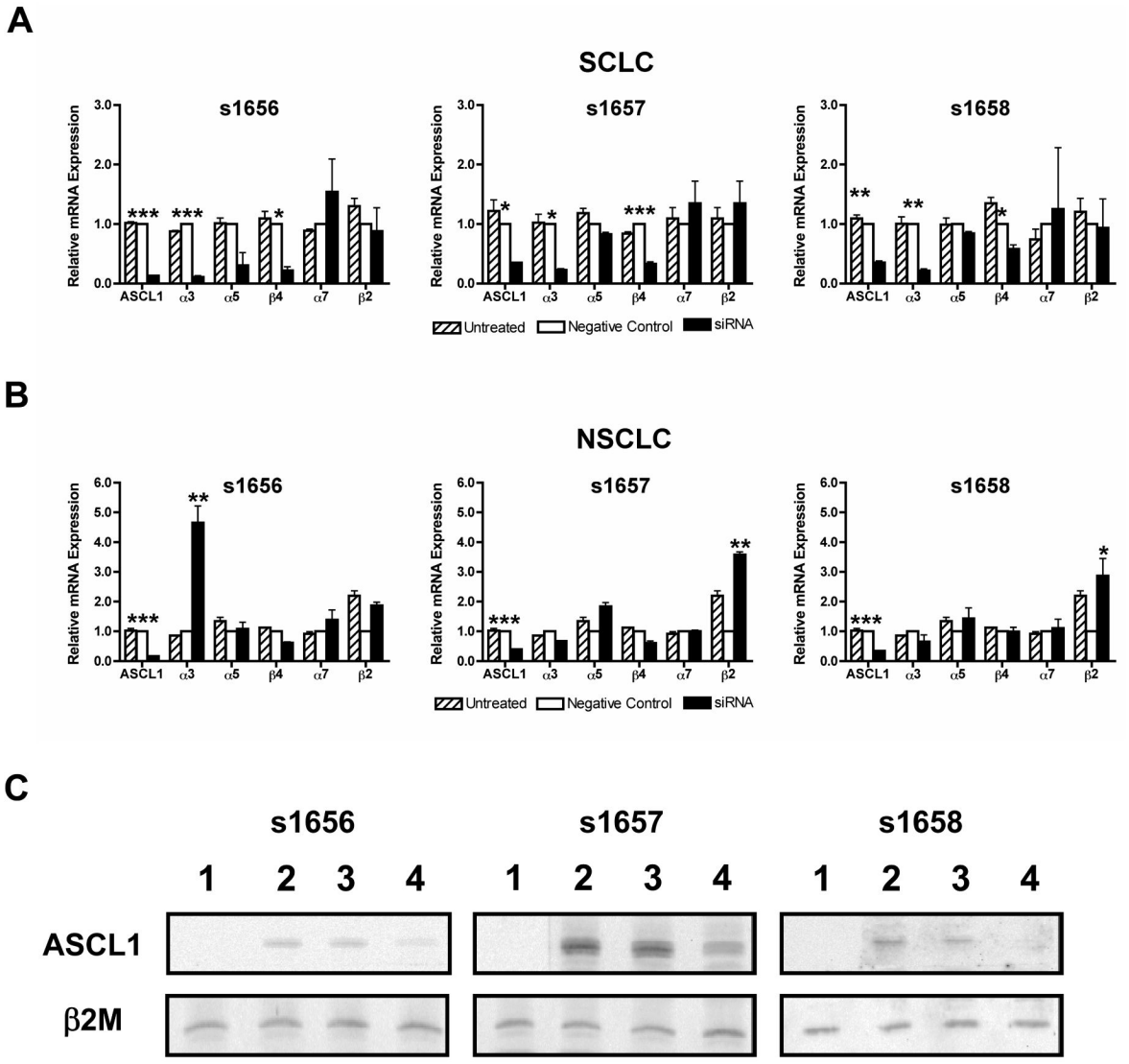

FIGURE 6.

Knockdown of ASCL1 leads to a decrease in CHRNA3/B4 gene expression in SCLC (**A**) but not in NSCLC (**B**). ASCL1 knockdown was performed using three different siRNAs: s1656, s1657, and s1658. Changes in gene expression were determined using quantitative RT-PCR. Samples treated with a negative control siRNA (white boxes) were used as calibrator (expression levels in these samples were set at 1). Error bars indicate standard errors of means. Asterisks indicate statistical difference between negative control and siRNA-treated samples (* = p value < 0.05, **=p value < 0.01, *** = p value < 0.001). Decrease in ASCL1 protein expression upon knockdown was confirmed using Western blot analysis (**C**). β2-microglobulin (β2M) was used as loading control. Lane 1: No ASCL1 primary antibody; Lane 2: Untreated cells; Lane 3: Negative control siRNA; and Lane 4: siRNA-treated cells.

Table 1

Clinical Characteristics of Lung Samples

Specimen ID	Histology	Sex	Age	Stage	Smoking History
143C	SCLC	M	54	Extensive	+
985T	SCLC	M	71	T1N0M0	+
1662T	SCLC	M	59	Extensive	+
1090251A2	SCLC	M	57	NA*	NA
08-02-A280a	SCLC	M	61	T2N2MX	NA
06-11-A306aa	SCLC	F	72	NA	NA
MAD09-131T	SCLC	M	53	T2N0MX	+
350T	Large Cell	M	51	T2N0MX	+
808T	Large Cell	F	45	T2N1M0	+
849T	Large Cell	M	74	T3N1M0	+
1722T	Large Cell	F	74	T2N0MX	NA
MAD02-1005T	Large Cell	M	76	T2N0MX	+
MAD05-467T	Large Cell	F	61	T1N0MX	+
MAD07-597T	Large Cell	M	73	T1N0MX	+
MAD07-661T	Large Cell	M	71	T1N2MX	+
MAD07-809T	Large Cell	F	70	T2N0MX	+
MAD08-469T	Large Cell	M	61	T2N0MX	+
MAD08-638T	Large Cell	M	44	T2N1MX	+
Z4312A1E	Large Cell	M	NA	T2N2MX	NA
343T	Adenocarcinoma	M	62	T1N0MX	+
363T	Adenocarcinoma	F	56	T1N2MX	+
423T	Adenocarcinoma	M	64	NA	+
457T	Adenocarcinoma	M	64	T4N1MX	+
43089A1C	Adenocarcinoma	F	81	NA	NA
43464A1C	Adenocarcinoma	F	59	NA	+
43471A1C	Adenocarcinoma	F	26	T3N2MX	−
44833A1A	Adenocarcinoma	F	82	NA	+
45139A1D	Adenocarcinoma	F	61	NA	+
45151A3CA	Adenocarcinoma	NA	NA	NA	NA
45514A1A	Adenocarcinoma	NA	NA	NA	NA
45607A1BA	Adenocarcinoma	M	78	NA	+
46127A1BA	Adenocarcinoma	M	77	NA	+
46244A1A	Adenocarcinoma	F	66	T4N1MX	NA
46598A1A	Adenocarcinoma	M	67	NA	+
1081210A1	Adenocarcinoma	NA	NA	NA	NA
1090694A1	Adenocarcinoma	F	66	NA	NA
08-04-A123A	Adenocarcinoma	M	67	NA	NA

Specimen ID	Histology	Sex	Age	Stage	Smoking History
Z4364A1A	Adenocarcinoma	F	NA	T2N1MX	NA
258T	Squamous	F	74	T1N0MX	+
318T	Squamous	M	81	T2N1MX	+
43312T	Squamous	F	76	NA	NA
43751T	Squamous	NA	NA	T2N1MX	NA
43057A1I	Squamous	M	68	NA	+
45843A1F	Squamous	M	41	NA	NA
46215A1F	Squamous	F	75	NA	NA
46830A1A	Squamous	NA	NA	NA	NA
1082331B2	Squamous	F	69	NA	NA
1090147A2	Squamous	M	72	NA	NA
3081395A3	Squamous	F	51	NA	NA
3081583A5	Squamous	F	70	NA	NA
3090415A2	Squamous	M	56	T2N0MX	NA
08-01-A310A	Squamous	M	61	NA	NA
08-02-A290A	Squamous	F	58	T2N0MX	NA
08-05-A023B	Squamous	F	75	NA	NA
08-07-A078A	Squamous	F	65	T2N0MX	NA
08-08-A097B	Squamous	M	58	T2N1MX	NA
08-09-A190A	Squamous	M	58	NA	NA
08-11-A001B	Squamous	M	63	T1N1MX	NA
08-12-A011A	Squamous	M	74	T1N0MX	NA
09-03-A012B	Squamous	M	70	T1N2MX	NA
09-04-A019A	Squamous	M	75	NA	NA
MAD06-482T	Squamous	M	65	T1NXMX	+
MAD06-552T	Squamous	M	73	T1NXMX	+
MAD06-597T	Squamous	F	73	T1N0MX	+
MAD06-603T	Squamous	F	71	T1N0MX	+
MAD06-625T	Squamous	M	74	T2N1MX	+
Z3770A1A	Squamous	NA	NA	T3N0MX	NA
Z4129A1D	Squamous	M	NA	T2N1MX	NA
Z4363A1A	Squamous	M	NA	T2N1MX	NA
Z4640A1A	Squamous	F	NA	T2N0MX	NA

* NA – information not available