Aberrant DNA Methylation Links Cancer Susceptibility Locus 15q25.1 to Apoptotic Regulation and Lung Cancer

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Abstract
Nicotinic acetylcholine receptor (nAChR) genes form a highly conserved gene cluster at the lung cancer susceptibility locus 15q25.1. In this study, we show that the CHRNA3 gene encoding the nAChRα3 subunit is a frequent target of aberrant DNA hypermethylation and silencing in lung cancer, whereas the adjacent CHRNβ4 and CHRNA5 genes exhibit moderate and no methylation, respectively. Treatment of cancer cells exhibiting CHRNA3 hypermethylation with DNA methylation inhibitors caused demethylation of the CHRNA3 promoter and gene reactivation. Restoring CHRNA3 levels through ectopic expression induced apoptotic cell death. Small hairpin RNA–mediated depletion of nAChRα3 in CHRNA3-expressing lung cancer cells elicited a dramatic Ca2+ influx response in the presence of nicotine, followed by activation of the Akt survival pathway. CHRNA3-depleted cells were resistant to apoptosis-inducing agents, underscoring the importance of epigenetic silencing of the CHRNA3 gene in human cancer. In defining a mechanism of epigenetic control of nAChR expression in nonneuronal tissues, our findings offer a functional link between susceptibility locus 15q25.1 and lung cancer, and suggest nAChRs to be theranostic targets for cancer detection and chemoprevention. Cancer Res; 70(7): 2779-88. ©2010 AACR

Introduction

Nicotinic acetylcholine receptors (nAChR), encoded by the nicotinic acetylcholine receptor encoding (CHRN) genes clustered at the lung cancer susceptibility locus 15q25 (1–4), are prototypic ligand-gated ion channels that are activated by endogenous agonists (acetylcholine and choline) and the exogenous compound nicotine. Activation of nAChR induces the opening of nonselective cation channels and voltage-gated Ca2+ channels (5), and the principles of nAChR function have been preserved in evolution (6). CHRN genes are expressed in both neuronal and nonneuronal tissues, suggesting that nAChRs may play an important role in processes other than synaptic transmission. Indeed, apart from their classic role at neuromuscular junctions, nAChRs have also been implicated in the regulation of cellular processes such as proliferation, cell-cell interaction, and cell death (7–10), although underlying mechanisms remain poorly understood.

Different nAChR subunits are expressed in normal lung tissues, and nicotine exposure has been theorized as influencing the expression of nAChR subunit genes (9, 10). The nAChR subunit composition in turn further regulates the function and pharmacology of nAChR; however, the exact mechanisms governing expression and assembly of nAChRs in normal lung epithelium and lung cancer tissues are largely unknown (7, 8, 11). nAChRs are thought to be heteropentamers composed of combinations of different α and β subunits, encoded by a conserved family of at least 12 CHRN genes (CHRNA2–α10 and CHRNβ2–β4). Although several single nucleotide polymorphisms (SNP) spanning the CHRNA3–CHRNβ4–CHRNA5 gene cluster have been associated with lung cancer incidence and susceptibility, only SNP RS16969968 has been identified to result in the frequent amino acid substitution Asn398Asp in the CHRNA5 gene (12).

Interestingly, it was found that lung cancer cells may express a distinct pattern of nAChR subunits (13), and activation of nAChR receptors and nAChR subunit composition may regulate critical cellular processes in nonneuronal tissues (7). For example, nicotine, at concentrations found in active smokers, was shown to inhibit apoptosis in lung cancer cells (14), whereas the activation of nAChRs in lung epithelial cells triggered stimulation of cell proliferation (14, 15). These results suggest that deregulation of CHRN gene expression and changes in nAChR functional states may lead to disruption of normal cell proliferation and cell death in normal lung tissues. Watanabe and colleagues (16) found that the nAChRα4 gene promoter exhibit differential patterns of DNA methylation in murine nonneuronal tissues (liver, muscle, and brain), suggesting that epigenetic mechanisms...
may be responsible for the tissue-specific expression of the nAChR genes. However, little is known on the extent of dere
egulation of nAChR-encoding genes in human cancer and the possible mechanisms underlying disruption of nAChR function in lung tissues.

In this study, we tested the hypotheses that expression of nAChR-encoding genes clustered at the 15q25.1 locus may be under epigenetic regulation and that epigenetic silencing of CHRN genes may contribute to lung cancer. We present evidence indicating that the CHRNA3 gene exhibits frequent DNA hypermethylation in lung tumors, and that these epigenetic changes are associated with unscheduled gene silencing and abrogation of cell death.

Materials and Methods

Tumor samples. Lung cancer samples and blood control samples were obtained from a case-control study on lung cancer conducted at Cancer Research Centre, Moscow (Russia), as a part of a larger multicenter case-control study coordinated by the International Agency for Research on Cancer (2, 17). Both lung cancer samples and blood control samples were described elsewhere (2, 17). Informed consent was obtained from all patients, and the study was approved by the relevant institutional review committee.

Cell lines, culture conditions, and transfections. Human lung cancer cell lines used were maintained in standard medium under conditions recommended by the American Type Culture Collection. Transient transfections for these cells were carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Mammalian expression constructs containing full-length cDNAs of the CHRNA3, CHRNβ4, and CHRNA5 genes, under control of cytomegalovirus (CMV) promoter, were kindly provided as a gift from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). For inducible depletion of the CHRNA3 gene, H1299 and H1650 lung cancer cells were transfected with pTRIPZ plasmid encoding small hairpin RNAs (shRNA), and hence was used as a reporter for morphometric measurement of DNA methylation levels in the promoter region of the CHRNA3, CHRNβ4, and CHRNA5 genes (Supplementary Table S2).

Gene expression analysis. Total RNA was extracted from different cell cultures using the RNaseasy Mini Kit (Qiagen) following the manufacturer’s protocol. The reverse transcriptase reaction was subjected to semiquantitative or quantitative PCR analysis (Supplementary Table S3). For quantitative PCR analysis, real-time quantitative PCR (RT-qPCR) was performed in quadruplicate with the MXP3000 real-time PCR system (Stratagene) using the Universal Probe Library System (Roche) and TaqMan Universal PCR Master Mix according to the manufacturer’s instructions. Primers and probes were designed using Universal Probe Library Assay Design Center (Roche; Supplementary Table S3).

Demethylating drug treatment. Cells were incubated in culture medium containing 5 μmol/L of 5-aza-2’-deoxycytidine (Azac; Sigma) or 50 μmol/L RG108 (a kind gift from Deutsches Krebsforschungszentrum) for 6 d with daily medium change. The effects of demethylating agents on the methylation and expression levels of the candidate CHRN genes were monitored by pyrosequencing and RT-qPCR, respectively.

Annexin V–FITC assay. Cells in the early stages of apoptosis were detected using the ApoAlert Annexin V–FITC apoptosis kit (Clontech), following the manufacturer’s protocol.

Western blot analysis. Western blotting was carried out essentially as described previously (19). Blots were probed with anti-CHRNA3 antibody (ab55773; Abcam) at a 1:1,000 dilution. Antibodies against native and phospho-Akt, -p42/p44, -p90, NF-κB, and poly(ADP-ribose) polymerase (PARP) were all from Cell Signaling Technology. Mouse monoclonal antibody (Clone C4; MP Biomedicals, France) to β-actin was used as loading control.

Immunohistochemical analysis. The avidin-biotin peroxidase complex method was used for immunohistochemical analysis of α3 nAChR. After deparaffinization, unstained slides were treated with microwave heating in antigen retrieval solution for 10 min. Anti-CHRNA3 antibody (a gift from Prof. Lindstrom) was applied at a 1:50 dilution.

Ca2+ influx microscopy. The effect of nicotine on Ca2+ influx was monitored essentially as previously described (20). Briefly, the cells were seeded at concentrations of 1 × 10^5/mL into a four-chamber glass (Labtek). Cells positive for induction were tracked by RGF reporter gene expression. Dye-loaded lung cancer cells were treated with 10 mmol/L nicotine and examined using a confocal argon ion laser scanning unit (LSM 410, class 3B; Carl Zeiss).

Statistical analysis. Statistical analysis was carried out essentially as described previously (17), using SPSS software version 16.1. The correlation between immunohistochemical staining intensity scores and DNA methylation level was determined using Spearman’s nonparametric correlation analysis. ANOVA, followed by Newman-Keuls’ test was used to determine mean differences between different groups of replicates, to find differences within the methylation and expression levels. Mean differences for apoptotic responses between mock-transfected and transfected cells.
with different CHRN gene constructs were calculated using Student’s t test. P < 0.05 or P < 0.01 was considered statistically significant.

Results

CHRNα3 and CHRNβ4 genes exhibit lung tumor-specific hypermethylation. To test the hypothesis that nAChR-encoding genes may be regulated epigenetically, we quantitatively determined DNA methylation levels at multiple CpG sites in the promoter of three nAChR-encoding genes (CHRNα3, CHRNβ4, and CHRNα5) clustered at the locus in chromosome region 15q25.1 (Fig. 1A). All the CpG sites included in the analysis are in the bona fide CpG islands and located at 377 to 403 bp, 3 to 49 bp, and 277 to 316 bp upstream of the transcription start sites of the α3, β4, and α5 subunit encoding CHRN genes, respectively. We combined pyrosequencing, the technique allowing quantitative analysis of multiple CpG sites, with a series of lung tumors (n = 142) and control blood samples (n = 164; Fig. 1B). Results of the pyrosequencing analysis of CHRNα3, CHRNβ4, and CHRNα5 genes in lung tumors and blood samples are shown in Fig. 1C and Supplementary Fig. S1. The results revealed that CHRNα3 exhibited significantly higher levels of DNA methylation in lung tumors (Fig. 1C; Supplementary Fig. S1). CHRNα5 was virtually unmethylated (mean methylation levels <5%; Fig. 1), whereas CHRNβ4 exhibited intermediate methylation levels (Fig. 1C). These results indicate that CHRNα3 and CHRNβ4 exhibit a highly significant and moderate hypermethylation, respectively, whereas CHRNα5 remains unmethylated in lung tumors.

We next analyzed methylation levels of CHRNα3, CHRNβ4, and CHRNα5 in normal-appearing adjacent lung tissues and found that levels of DNA methylation in CHRNα3 and CHRNβ4 in adjacent tissues were comparable with those in blood samples, but significantly lower compared with the methylation levels seen in lung cancer (Fig. 1D). These results confirm the notion that CHRNα3 and CHRNβ4 exhibit tumor-specific hypermethylation.

Analysis of DNA methylation frequency (defined as the percentage of tumor samples with methylation levels >90% of quantile levels in nontumor adjacent lung tissue samples) showed that CHRNα3 and CHRNβ4 genes were frequently hypermethylated (in 37% and 24% patient samples, respectively), whereas hypermethylation of the CHRNα5 gene was relatively infrequent (7%) in lung tumors (Table 1; Supplementary Fig. S1).

Hypermethylation of CHRNα3 is associated with its silencing in lung cancer. Because aberrant DNA hypermethylation of the gene promoter can lead to downregulation of gene expression, we next determined whether levels of CHRN methylation correlate with expression of the gene. Immunohistochemical analysis using antibody against CHRNα3 protein revealed that the tumor samples with CHRNα3 hypomethylation showed lower staining intensities compared with those with less methylation (significant correlation; r = −0.66), indicating that samples having hypermethylation of CHRNα3 gene have less expression levels of the nAChRα3 protein (Fig. 2A and B). These results suggest that hypermethylation of CHRNα3 gene may result in partial or complete silencing of the gene. Interestingly, a similar association between staining intensities of nAChRβ4 protein and the CHRNβ4 methylation levels in lung cancer was not observed (data not shown).

We next treated four lung cancer cell lines (A549, H1299, H1975, and H1650) with two different demethylating drugs, AzaC and RG108 (21), and examined both the expression and methylation levels of the CHRN genes. Although there were notable differences in DNA methylation levels between cell lines, the observed methylation levels in the cell lines were comparable with those observed for the individual genes in different lung tumor samples (Figs. 1C and 2C). Treatment with either of the two DNA methylation inhibitors resulted in a modest but consistent demethylation of CHRNα3 gene in cancer cell lines. Importantly, CHRNα3 responded with higher levels of expression following treatment with a demethylating agent (Fig. 2C), consistent with previous reports that DNA methylation inhibition can effectively reactivate transcriptionally silent genes associated with unscheduled hypermethylation (22). In contrast, demethylation treatment failed to significantly increase expression levels of CHRNβ4 and CHRNα5 despite a noticeable reduction in methylation levels in these genes (Fig. 2C), suggesting that methylation levels of these genes found in these cancer cells do not interfere with their expression. Therefore, DNA hypermethylation appears to specifically target the expression of the CHRNα3 gene, whereas the CHRNβ4 and CHRNα5 genes may not be deregulated through aberrant DNA methylation in lung cancer cells.

Restoration of expression or overexpression of CHRNα3 induces apoptotic cell death. To understand the functional impact of deregulated expression of the CHRNα3 gene on cellular processes, we sought to restore expression of CHRNα3 gene in cancer cell lines. To this end, CMV promoter–driven CHRNα3, CHRNβ4, or CHRNα5 expression vectors or an empty vector (control) were transfected into H1975 cells, wherein high levels of the CHRNα3 promoter methylation were found to result in lower expression of the gene (Fig. 2C), and examined their expression. Reverse transcription-PCR (RT-PCR) analysis of transfected cells revealed high levels of expression of the corresponding CHRN genes compared with the mock-transfected control (Fig. 3A). In addition, Western blot analysis of cell lysates from CHRNα3-transfected cells showed significantly higher levels of nAChRα3 protein levels compared with the mock-transfected cells (Fig. 3A).

To test the effect of increased expression of CHRN genes on cell death, H1975 cells were mock-transfected or transfected with increasing concentrations of plasmids expressing CHRNα3, CHRNβ4, or CHRNα5, and cell death fraction after staining with FITC-conjugated Annexin V (whose binding to externalized phosphatidyl-serine on cell membrane is an early indicator of apoptosis) was analyzed. As shown in Fig. 3B and C, CHRNα3 transfection resulted in a dose-dependent increase in apoptotic fraction, whereas transfection of cells with the vector expressing CHRNβ4 and CHRNα5 genes failed to induce appreciable levels of apoptotic cell death.
Figure 1. DNA methylation analysis of the CHRN genes clustered at the 15q25.1 locus in lung cancer. A, schematic representation of the chromosomal arrangement of the 15q25.1 loci containing three nAChR genes. Location of the analyzed sequence and sets of primers for each gene analyzed are indicated by gray boxes and arrows, respectively. B, representative pyrograms of all three CHRN genes obtained from the pyrosequencing assays used to quantitatively determine the DNA methylation status across multiple CpG sites. C, box plots of the results obtained by the analysis of individual genes and CpG sites. DNA methylation levels obtained by analyzing the mean methylation levels of all the CpG sites for CHRNA3, CHRNβ4, and CHRNA5 gene in tumor tissues (T) and blood samples (B). D, analysis of methylation of CHRN genes in lung tumors and normal-appearing adjacent tissues. Levels of methylation obtained for CHRNA3, CHRNβ4, and CHRNA5 genes in blood (B), lung tumors (T), and normal-appearing adjacent tissues (AT). The statistical significance for differential methylation in tissues analyzed was calculated using Newman-Keuls' test.
death (Fig. 3B and data not shown). Increased apoptotic cell death in cells transfected with CHRNA3 was further verified by flow cytometric analysis of cell cycle profiles following propidium iodide staining, which revealed significantly higher sub-G1 population in CHRNA3-transfected cells (76%) compared with mock-transfected cells (8.5%; Fig. 3C), and the induction of proteolytic cleavage of PARP (Fig. 3C), a hallmark of apoptosis. These results indicate that restoration of nAChRα3 expression in cancer cells induces cell death, and that cell death triggered by ectopic expression of CHRNA3 gene is mediated by the apoptotic pathway. Induction of apoptosis following restoration of CHRNA3 expression was further found to be associated with activated mitogen-activated protein kinase (MAPK) signaling pathway (as judged by a dose-dependent increase in phosphorylation levels of both p44/p42 MAPK and p38 MAPK proteins), whereas activation of the NF-κB signaling pathway was not observed in these cells (Fig. 3D). These results suggest that epigenetic silencing of CHRNA3 may impair apoptotic cell death during tumor development, and that the restoration of its expression may sensitize cancer cells to apoptotic death through activation of MAPK stress signaling.

**Knockdown of CHRNA3 gene deregulates nAChR-associated signaling and impairs cell death response.** To examine the physiologic significance of the methylation-mediated silencing of CHRNA3 gene, we used shRNAs to inhibit the expression of the CHRNA3 gene in human cancer cells, and tested the function of nAChR receptors and apoptotic proficiency. Two lung cancer cell lines, H1650 and H1299, both of which exhibit low methylation levels and express appreciable levels of nAChRα3 (Figs. 2C and 4A), were stably transfected with a doxycycline-inducible shRNA expression construct targeting CHRNA3. Successful silencing of the target gene in the presence of doxycycline (+Dox) was confirmed at both transcript (data not shown) and protein levels (Fig. 4A). We next tested the effect of nAChRα3 knockdown on the function of nAChRs, the activation of which is known to induce opening of nonselective cation and voltage-gated Ca2+ channels leading to the downstream cellular signaling events (8, 9). As shown in Fig. 4B, nAChRα3-depleted cells (+Dox) exhibited a significantly higher Ca2+ influx response upon nicotine exposure, compared with the nAChRα3-expressing control cells (−Dox), indicating that CHRNA3 depletion triggers hyperactivation and dramatic opening of Ca2+ channels in the presence of nicotine.

To test whether cholinergic signaling by nicotine triggers a survival signaling pathway in nAChRα3-deficient cells, we investigated MAP/extracellular signal-regulated kinase (MEK) and Akt signaling, two pathways known to be activated by nicotine/cholinergic signaling through AChR7 receptors (9, 15, 23–26). Incubation of cells in the presence of nicotine resulted in a higher levels of phosphorylation of Akt and p42/p44 proteins in nAChRα3 knockdown cells compared with nAChRα3-expressing cells (Fig. 4C), indicating that a higher Ca2+ influx in nAChRα3-depleted cells is followed by activation of the MEK and Akt signaling cascade. Together, these results show that knockdown of CHRNA3 results in an activation of cholinergic signaling by nicotine and triggers the MEK/Akt survival signaling pathway.

To further explore the effects of CHRNA3 inactivation on cell survival and apoptosis proficiency, H1299 and H1650 cells expressing inducible shRNA were treated with different apoptosis-inducing agents in the absence or presence of doxycycline, and apoptotic fraction (Annexin V–positive cells) was determined by flow cytometry. Whereas both CHRNA3-expressing and CHRNA3-knockdown cells in the absence of apoptosis-inducing agents exhibited comparable levels of Annexin V–positive cells, suggesting that loss of CHRNA3 does not lead to spontaneous cell death, CHRNA3-depleted cells treated with apoptosis-inducing agents exhibited significantly lower apoptotic responses to etoposide, cycloheximide, and doxorubicin (Fig. 4D). These findings support the notion that downregulation of CHRNA3 gene expression impairs apoptotic proficiency.

**Discussion**

The present study revealed that the 15q25.1 locus may be under epigenetic regulation and that its deregulation may contribute to the development of lung cancer. Although CHRNA3, CHRNA4, and CHRNA5 are clustered together at

### Table 1. Frequency of CpG hypermethylation in lung tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Methylation levels (maximum/minimum/average)</th>
<th>Methylation level cutoff*</th>
<th>Hypermethylated tumor samples† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHRNA3</td>
<td>64.83%/0%/21.14%</td>
<td>22.67%</td>
<td>52/142 (36.6%)</td>
</tr>
<tr>
<td>CHRNA4</td>
<td>51.14%/0%/13.36%</td>
<td>15.03%</td>
<td>30/124 (24.2%)</td>
</tr>
<tr>
<td>CHRNA5</td>
<td>7.51%/0%/1.57%</td>
<td>5.72%</td>
<td>7/103 (6.8%)</td>
</tr>
</tbody>
</table>

*The 90% percentile value for the surrounding nontumor lung tissue samples.
†Samples with methylation levels above the quantile representing the upper 90% of methylation in the surrounding nontumor lung tissue samples.
the same locus, they exhibit differential susceptibility to aberrant methylation. Interestingly, we found that elevated CHRNα3 methylation, in contrast to hypermethylation of the CHRNα3 gene, failed to inhibit gene expression. However, as levels of CHRNα3 methylation in lung tumors were markedly lower than those of CHRNα3, it is therefore possible that methylation levels of CHRNα3, although increased, were insufficient to induce significant silencing of the gene. Alternatively, lack of transcriptional repression may be due to lower CpG density in the CHRNα3 promoter (57 CpG sites in the island) compared with the CHRNα3 promoter (137 CpG sites in the island; http://genome.ucsc.edu/cgi-bin/hgGateway), consistent with the notion that the density of methylated CpG sites is a critical factor for transcriptional silencing and that repression by DNA methylation requires a high density of methylated cytosines (27).

The finding that restoration of CHRNα3 expression in lung cancer cells harboring silenced endogenous CHRNα3 gene induces apoptosis suggests that silencing of CHRNα3 expression may abrogate susceptibility to cell death and contribute to cancer development. Consistent with this notion, CHRNα3 downregulation using an shRNA approach in CHRNα3-expressing cells leads to apoptotic defects in response to different apoptosis-inducing agents. These results argue that silencing of CHRNα3 expression in nonneuronal tissues (such as normal lung epithelium) may abrogate physiologic cell death and promote cell survival. Transfection experiments using varying concentrations of CHRNα3 expression vector resulted in a dose-dependent increase in apoptotic fraction, suggesting that CHRNα3 gene dosage and its transcriptional control play an important role in cellular susceptibility to apoptosis. The mechanism by which CHRNα3 silencing contributes to cellular evasion of apoptosis appears to involve nAChR receptors whose activation induces opening of voltage-gated Ca²⁺ channels and the downstream prosurvival signaling events (8, 9). This notion is supported by our results showing a significantly higher Ca²⁺ influx in CHRNα3-depleted cells followed by activation of MEK and Akt survival signaling pathways, inhibition of apoptosis, and promotion of survival. However, it remains unclear how inactivation of CHRNα3 gene expression and consequent depletion of nAChRα3 subunit may result in aberrant function of the nAChR receptors. The genes encoding nAChR subunits are thought to be under a coordinated transcriptional regulation (28, 29), and nAChRα3, nAChRα5, and nAChRβ4 subunits assemble together to form functional nAChR receptor complexes. Loss of one subunit of heteromeric nAChRs may affect the activation properties of the receptors and the cellular response to different receptor agonists/antagonists, including nicotine; therefore, higher Ca²⁺ influx in nAChRα3 subunit-depleted cells may result from altered nicotine binding affinity and activation properties of nAChRs lacking the nAChRα3 subunit. Alternatively, these altered properties of nAChRs in nAChRα3-depleted cells may also result from the overrepresentation of other subtype-containing nAChRs. Because nAChRα7 receptors are the primary receptor types that mediate the proliferative effects of nicotine in the tumor cells (15) and are also considered to be the major stimulator of cancer development and progression (30, 31), silencing of the nAChRα3-encoding gene may result in overrepresentation of other nAChR subunits (notably nAChRα7 and nAChRα5), which may stimulate cell survival and provide a proliferation advantage to tumor cells. Based on our results, we propose a model (Supplementary Fig. S2) in which the CHRNα3 gene, apart from its classic regulatory role in neurotransmission, plays an important role in the regulation of cell survival/cell death of nonneuronal cells (i.e., lung epithelial cells).

In summary, our results provide mechanistic insights into the events through which silencing of the CHRN gene may deregulate cellular response to apoptotic stimuli in nonneuronal tissue and promote tumor development, providing a functional link between susceptibility locus 15q25.1 and lung cancer. However, it should be noted that our results indicate that in lung cancer tumors (and not in surrounding nontumor lung tissue of same patients), the 15q25.1 locus is aberrantly methylated, resulting in silencing of the CHRNα3 gene, suggesting that irrespective of the known risk factor exposure, hypermethylation of CHRNα3 gene is a common and tumor tissue–specific phenomenon. Although further studies are required to discover possible causes of selective hypermethylation at 15q25.1 locus, the information provided may open new research avenues to investigate different epigenetic mechanisms by which nicotine deregulation of acetylcholine receptors in nonneuronal tissues may contribute to cancer development and progression. Our study also reinforces the interest in nAChRs and

Figure 2. Expression of CHRN genes and its association with DNA hypermethylation. A, immunohistochemical analysis showing methylation level–dependent expression of nAChRα3 among different lung tumor samples. Representative micrographs of immunohistochemical analysis show high (top) and low (bottom) expression of nAChRα3 in the two representative lung cancer samples. Dark staining is indicative of the presence of nAChRα3 protein. Tissue is counterstained to indicate cell nuclei (blue staining). B, comparison of DNA methylation and expression levels of nAChRα3 in individual lung tumor samples. Intensity of staining was scored from 0 to 4 as described in Materials and Methods. A significant inverse correlation between CHRNα3 methylation and nAChRα3 expression levels was observed (Pearson correlation coefficient, r = -0.66). C, the effect of treatment with DNA-demethylating drugs on CHRN gene expression in lung cancer cells. Four different lung cancer cell lines were treated with indicated doses of two different demethylating drugs (Azac and RG108) for 6 d, and mRNA and methylation levels of CHRNα3, CHRNβ4, and CHRNα5 were analyzed. The gene expression levels were determined by quantitative RT-PCR assays specific for each CHRN gene, and DNA methylation levels were measured quantitatively by pyrosequencing. Black bars and grey circles represent mean expression and methylation values, respectively. The error bars represent SEM. For a given cell line, statistically significant (P < 0.05) methylation or expression values were calculated by comparing the treated groups with the vehicle treated group (control). *- statistically significant expression or methylation values (P < 0.05).
Figure 3. Restoration of expression of \( \text{CHRNa3} \) in cancer cells induces apoptosis. A, H1975 cells were transfected with empty vector (mock) or the vector containing the cDNA of \( \text{CHRNa3}, \text{CHRNB4}, \) and \( \text{CHRND5} \) genes and expression levels of individual genes were determined by RT-PCR analysis (top). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels are used as a loading control. Western blot analysis of \( \text{CHRNa3} \) expression (bottom). H1975 cells were transfected with empty vector (mock) or the indicated concentrations of the \( \text{CHRNa3} \)-expressing vector, and nAChR\( \alpha3 \) protein levels were determined by Western blotting. Equal loading was verified by anti-actin antibody. B, H1975 cells were transfected with indicated concentrations of the \( \text{CHRNa3}, \text{CHRNB4}, \) and \( \text{CHRND5} \) expression vector, and the apoptotic fraction was determined 36 h posttransfection by flow cytometry following staining with Annexin V. The results are given as the mean ± SD for triplicate samples. Data are from one of two independent experiments. *, statistical significance (\( P < 0.05 \)) calculated by comparing mock-transfected samples with all other sample types in the same group. C, H1975 cells were mock-transfected (empty vector) or transfected with the vector containing the cDNA of \( \text{CHRNa3} \), and apoptotic fraction (Annexin V staining; top) and sub-G1 population (middle) were determined by flow cytometry. Western blot analysis of PARP cleavage after the reexpression of \( \text{CHRNa3} \) in H1975 cells. Full-length PARP (113 kDa) and cleaved fragments (89 kDa) are indicated. D, restoration of \( \text{CHRNa3} \) expression is associated with activated MAPK/p38 stress signaling. H1975 cells were mock-transfected (empty vector) or transfected with the vector containing the \( \text{CHRNa3} \) expression vector; 36 h posttransfection, cells were harvested and analyzed by Western blotting using different antibodies.
Figure 4. Effect of the CHRNα3 gene knockdown on nAChR signaling and apoptotic proficiency of lung cancer cells. A, H1650 and H1299 cells were stably transfected with an inducible shRNA expression vector targeting CHRNα3 gene, and the efficiency of shRNA-mediated silencing was revealed by Western blots using anti-nAChRα3 antibody. To silence the CHRNα3 gene, expression of shRNA was induced with 1 μg/ml doxycycline (+Dox) for 48 h. B, analysis of nicotine-induced Ca2+ influx in H1299 cells expressing different levels of nAChRα3. H1299 cells were grown in the presence (+Dox) or absence (−Dox) of doxycycline, and following addition of 10 mmol/L nicotine (time point, 0 s), the imaging of intracellular Ca2+ was performed using the Ca2+ indicator fluo-4 (10 μmol/L) and confocal laser microscopy (top). RGFP expression is under the control of the same doxycycline-inducible promoter as the α3-shRNA and is thus used as a reporter to shRNA expression. Plot comparing the quantified fluorescent intensity changes in CHRNα3-depleted (+Dox) and CHRNα3-expressing (−Dox) cells (bottom). C, knockdown of CHRNα3 expression triggers activation of the Akt-signaling pathway. H1299 cells were treated with nicotine (10 mmol/L) for 48 h following prior incubation in the presence (+Dox) or absence (−Dox) of doxycycline for 48 h. Phosphorylation of Akt (PO4-Akt) and p42/44 (PO4-p42/44), and native Akt and p42/44 protein levels in total protein lysates were monitored by Western blot analysis. Equal loading was verified by anti-actin antibody. D, knockdown of CHRNα3 gene impairs cell death response. H1650 and H1299 cells stably transfected with inducible CHRNα3 shRNA were incubated in the presence or absence of doxycycline for 24 h and treated with etoposide, cycloheximide, or dexamethasone (100 μmol/L) for a further 24 h. Cells were then stained with Annexin V/propidium iodide, and apoptotic fraction was analyzed by fluorescence-activated cell sorting. Shown are mean values (±SD) from three different experiments. *, statistical significance calculated using Student’s unpaired t test (P < 0.05; two-tailed).
the genes encoding as markers for cancer detection, clinical predictors, and potential chemopreventive targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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