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Aberrant DNA methylation links cancer susceptibility locus 15q25.1 to apoptotic regulation and lung cancer

Anupam Paliwal¹, Thomas Vaissière¹, Annette Krais¹, Cyrille Cuenin¹, Marie-Pierre Cros¹, David Zaridze², Anush Moukeria², Paolo Boffetta³, Pierre Hainaut¹, Paul Brennan¹, and Zdenko Herceg¹

¹IARC, Lyon, France

²Cancer Research Center, Moscow, Russia

³The Tisch Cancer Institute, Mount Sinai School of Medicine, New York, and International Preventive Research Institute, Lyon, France

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 *CHRN* α 3 gene encoding nAChR α 3 subunit is a frequent target of aberrant DNA hypermethylation and silencing in lung cancer, whereas the adjacent *CHRN* β 4 and *CHRN* α 5 genes exhibit moderate and no methylation, respectively. Treatment of cancer cells exhibiting *CHRN* α 3 hypermethylation with DNA methylation inhibitors caused demethylation of the *CHRN* α 3 promoter and gene re-activation. Restoring *CHRN* α 3 levels through ectopic expression induced apoptotic cell death. shRNA-mediated depletion of nAChR α 3 in *CHRN* α 3-expressing lung cancer cells elicited a dramatic Ca²⁺ influx response in the presence of nicotine, followed by activation of the Akt survival pathway. *CHRN* α 3-depleted cells were resistant to apoptosis-inducing agents, underscoring the importance of epigenetic silencing of the *CHRN* α 3 gene in human cancer. In defining a mechanism of epigenetic control of nAChR expression in non-neuronal tissues, our findings offer a functional link between susceptibility locus 15q25.1 and lung cancer, and suggest nAChRs as theranostic targets for cancer detection and chemoprevention.

Keywords

epigenetic silencing; DNA methylation; nicotine acetylcholine receptor genes; apoptosis; lung cancer

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) encoded by the nicotinic acetylcholine receptor (*CHRN*) genes clustered at the lung cancer susceptibility locus 15q25 (1-4), are prototypic ligand-gated ion channels that are activated by the 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 non-neuronal tissues, suggesting that nAChRs may play an important role in

*Correspondence: Dr Zdenko Herceg, Epigenetics Group, IARC, 150 cours Albert Thomas, 69372 Lyon cedex 08, France, Phone: +33 4 72 73 83 98, Fax: +33 4 72 73 83 29, herceg@iarc.fr.

processes other than synaptic transmission. Indeed, apart from their classical 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 nicotinic acetylcholine receptor subunit genes (9,10). The nAChR subunit composition in-turn further regulates function and pharmacology of nAChR; however, the exact mechanisms governing expression and assembly of nAChRs in normal lung epithelium and lung cancer tissues is largely unknown (7,8,11). nAChRs are thought to be hetero-pentamers composed of combinations of different α and β subunits, encoded by a conserved family of at least 12 *CHRN* genes (*CHRNA2- α 10* and *CHRN β 2- β 4*). Although several single nucleotide polymorphisms (SNPs) spanning the *CHRNA3-CHRN β 4-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 non-neuronal tissues (Schuller, 2009). 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 *et al* found that the nAChR α 4 gene promoter exhibit differential patterns of DNA methylation in murine non-neuronal tissues (liver, muscle and brain), suggesting that epigenetic mechanisms may be responsible for the tissue-specific expression of the nAChR genes (16). However, little is known on the extent of deregulation of nAChR-encoding genes in human cancer and possible mechanisms underlying the disruption of nAChR function in lung tissues.

In this study, we tested the hypothesis 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 tumours, and that these epigenetic changes are associated with unscheduled gene silencing and abrogation of cell death.

MATERIALS AND METHODS

Tumour 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 used 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*, *CHRN β 4* and *CHRNA5* genes, under control of CMV promoter, were kindly provided as a

gift from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia). For inducible depletion of the *CHRNA3* gene, the H1299 and H1650 lung cancer cells were transfected with pTRIPZ plasmid encoding shRNA against the *CHRNA3* gene (V2LHS_112345; Thermo Fisher Scientific), followed by TET-ON induction of shRNA for 24–48 hours with 1.0 µg/ml doxycycline. In this plasmid, RFP is induced as part of the same transcript as the shRNA, and hence was used as a reporter for morphometric scoring.

DNA methylation analysis

DNA methylation analysis was performed by Pyrosequencing following DNA extraction and bisulfite conversion as previously described (17,18). For this analysis, we used 142 primary tumours of the lung and 164 blood samples (Supplementary Table 1). Adjacent nonmalignant lung tissues were available from 60 cases and were also used for the analysis. Bisulfite treatment was carried out with EZ Methylation-Gold kit (Proteogene) following the manufacturer's recommendations. The bisulfite-converted DNA was pyrosequenced using a pyrosequencing system (PSQ 96MA, Biotage). Pyrosequencing assays were designed for quantitative measurement of DNA methylation levels in the promoter region of the *CHRNA3*, *CHRNA4* and *CHRNA5* genes (Supplementary Table 2).

Gene expression analysis

Total RNA was extracted from different cell cultures using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The reverse transcriptase reaction was subjected to semi-quantitative or quantitative PCR analysis (Supplementary Table 3). For quantitative PCR analysis, the real-time quantitative PCR were 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 3).

Demethylating drug treatment

Cells were incubated in culture medium containing 5 µM of 5-Aza-2'-deoxycytidine (Sigma, St. Louis, MO) or 50 µM RG108 (a kind gift from Deutsches Krebsforschungszentrum, Heidelberg) for 6 days with daily medium change. The effects of demethylating agents on the methylation and expression levels of the candidate *CHRNA* genes were monitored by pyrosequencing and RT-qPCR, respectively.

Annexin V-FITC Assay

Cells in early stages of apoptosis were detected using the ApoAlert Annexin V-FITC apoptosis kit (Clontech), by following the manufacturers 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 1:1000 dilution. Antibodies against native and phospho- Akt, -p42/p44, -p38, NFκB and PARP were all from Cell Signaling Technology. Mouse monoclonal antibody (Clone C4; MP Biomedicals, France) to β-actin was used for 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 minutes. Anti-*CHRNA3* antibody (a gift from Prof. Lindstrom; University of Pennsylvania, Philadelphia) 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 4-chamber glass (Labtek). Cells positive for induction were tracked by RGFP reporter gene expression. Dye-loaded lung cancer cells were treated with 10mM nicotine and examined using a confocal argon ion laser scanning unit (LSM 410, class 3B; Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Statistical analysis was carried out essentially as described previously (17), using SPSS software, version 16.1. 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 with different *CHRN* gene constructs were calculated using Students 't' test. $P < 0.05$ or $P < 0.01$ was considered statistically significant.

RESULTS

CHRNA3 and CHRNβ4 genes exhibit lung tumour 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 (*CHRNA3*, *CHRNβ4* and *CHRNA5*) clustered at locus in chromosome region 15q25.1 (Figure 1A). All the CpG sites included in the analysis are in the *bona fide* CpG islands and located at 377-403 bp, 3-49 bp and 277-316 bp upstream of the transcription start sites of the $\alpha 3, \beta 4$ and $\alpha 5$ subunit encoding *CHRN* genes, respectively. We combined pyrosequencing, the technique allowing quantitative analysis of multiple CpG sites, with a series of lung tumours (n=142) and control blood samples (n=164) (Figure 1B). Results of the pyrosequencing analysis of *CHRNA3*, *CHRNβ4* and *CHRNA5* genes in lung tumours and blood samples are shown in Figure 1C and Supplementary Figure 1. The results revealed that *CHRNA3* exhibited significantly higher levels of DNA methylation in lung tumours (Figure 1C, Supplementary Figure 1). *CHRNA5* was virtually unmethylated (mean methylation levels <5%) (Figure 1), whereas *CHRNβ4* exhibited intermediate methylation levels (Figure 1C). These results indicate that *CHRNA3* and *CHRNβ4* exhibit a highly significant and moderate hypermethylation, respectively, whereas *CHRNA5* remains unmethylated in lung tumours.

We next analyzed methylation levels of *CHRNA3*, *CHRNβ4* and *CHRNA5* in normal appearing adjacent lung tissues and found that levels of DNA methylation in *CHRNA3* and *CHRNβ4* in adjacent tissues were comparable to those in blood samples, but significantly lower in comparison to the methylation levels seen in lung cancer (Figure 1D). These results confirm the notion that *CHRNA3* and *CHRNβ4* exhibit tumour-specific hypermethylation.

Analysis of DNA methylation frequency (defined as the percentage of tumour samples with methylation levels >90% quantile levels in non-tumour adjacent lung tissue samples), showed that *CHRNA3* and *CHRNβ4* genes were frequently hypermethylated (in 37% and 24% patient samples, respectively), while hypermethylation of the *CHRNA5* gene was relatively infrequent (7%) in lung tumors (Table 1; Supplementary Figure 1).

Hypermethylation of CHRNA3 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 *CHRNA3* protein revealed that the tumour samples with *CHRNA3* hypermethylation showed lower staining intensities in comparison to those with less methylation (significant correlation; $r=-0.66$), indicating that samples having hypermethylation of *CHRNA3* gene have less expression levels of the nAChR $\alpha 3$ protein (Figure 2A, B). These results suggest that hypermethylation of *CHRNA3* gene may result in partial or complete silencing of the gene. Interestingly, a similar association between staining intensities of nAChR $\beta 4$ protein and the *CHRN $\beta 4$* methylation levels in lung cancer was not observed (data not shown).

We next treated four lung cancer cell lines (A459, H1299, H1975 and H1650) with two different demethylating drugs, 5-Aza-2'-Deoxycytidine (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 to those observed for the individual genes in different lung tumour samples (Figure 2C and Figure 1C).

Treatment with either of two different DNA methylation inhibitors resulted in a modest but consistent demethylation of *CHRNA3* gene in cancer cell lines. Importantly, *CHRNA3* responded with higher levels of expression following treatment with a demethylating agent (Figure 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 *CHRN $\beta 4$* and *CHRNA5* despite a noticeable reduction in methylation levels in these genes (Figure 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 *CHRNA3* gene, whereas the *CHRN $\beta 4$* and *CHRNA5* genes may not be deregulated through aberrant DNA methylation in lung cancer cells.

Restoration of expression or overexpression of *CHRNA3* induces apoptotic cell death

To understand the functional impact of deregulated expression of the *CHRNA3* gene on cellular processes, we sought to restore expression of *CHRNA3* gene in cancer cell lines. To this end, CMV promoter-driven *CHRNA3*, *CHRN $\beta 4$* or *CHRNA5* expression vectors or an empty vector (control) were transfected into H1975 cells, wherein high levels of the *CHRNA3* promoter methylation were found to result in lower expression of the gene (Figure 2C), and examined their expression. RT-PCR analysis of transfected cells revealed high levels of expression of the corresponding *CHRN* genes in comparison to the mock-transfected control (Figure 3A). In addition, Western blot analysis of cell lysates from *CHRNA3*-transfected cells showed significantly higher levels of nAChR $\alpha 3$ protein levels in comparison to the mock-transfected cells (Figure 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 *CHRNA3*, *CHRN $\beta 4$* , or *CHRNA5*, 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 Figure 3B, C, *CHRNA3* transfection resulted in a dose-dependent increase in apoptotic fraction, whereas transfection of cells with the vector expressing *CHRN $\beta 4$* and *CHRNA5* genes failed to induce appreciable levels of apoptotic cell death (Figure 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 PI staining, which revealed significantly higher sub-G1 population in *CHRNA3*-transfected cells (76%) in comparison to mock transfected cells (8.5%) (Figure 3C), and the induction of proteolytic cleavage of PARP (Figure 3C), a hallmark of apoptosis. These results indicate that restoration of nAChR $\alpha 3$ 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 MAPK signaling pathway (as judged by a dose-dependent increase in phosphorylation levels of both p44/p42 MAP kinase and p38 MAP kinase proteins), whereas activation of the NF κ B signalling pathway was not observed in these cells (Figure 3D). These results suggest that epigenetic silencing of *CHRNA3* may impair apoptotic cell death during tumour development, and that the restoration of its expression may sensitize cancer cells to apoptotic death through activation of MAPK stress signalling.

Knockdown of *CHRNA3* gene deregulates nAChR-associated signalling and impairs cell death response

To examine the physiological significance of the methylation-mediated silencing of *CHRNA3* gene, we used small hairpin RNAs (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 nAChR α 3 (Figure 4A and Figure 2C), 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 (Figure 4A). We next tested the effect of nAChR α 3 knockdown on the function of nAChRs, activation of which is known to induce opening of non-selective cation and voltage-gated Ca²⁺ channels leading to the downstream cellular signalling events (8, 9). As shown in Figure 4B, nAChR α 3 depleted cells (+Dox) exhibited a significantly higher Ca²⁺ influx response upon nicotine exposure, in comparison to the nAChR α 3-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 signalling by nicotine triggers a survival signalling pathway in nAChR α 3-deficient cells we investigated MEK and Akt signalling, two pathways known to be activated by nicotine/cholinergic signalling through AChR α 7 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 nAChR α 3 knockdown cells compared to nAChR α 3-expressing cells (Figure 4C), indicating that a higher Ca²⁺ influx in nAChR α 3 depleted cells is followed by activation of the MEK and Akt signalling cascade. Together, these results show that knockdown of *CHRNA3* results in an activation of cholinergic signalling by nicotine and triggers the MEK/Akt survival signalling 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. While 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 doxorubicine (Figure 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*, *CHRN β 4* and *CHRNA5* are clustered together at the same locus, they exhibit differential

susceptibility to aberrant methylation. Interestingly, we found that elevated *CHRN* β 4 methylation, in contrast to hypermethylation of the *CHRNA*3 gene, failed to inhibit expression of the gene. However, as levels of *CHRN* β 4 methylation in lung tumours were markedly lower than those of *CHRNA*3, it is therefore possible that methylation levels of *CHRN* β 4, 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 *CHRN* β 4 promoter (57 CpG sites in the island) compared to the *CHRNA*3 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 *CHRNA*3 expression in lung cancer cells harbouring silenced endogenous *CHRNA*3 gene induces apoptosis suggests that silencing of *CHRNA*3 expression may abrogate susceptibility to cell death and contribute to cancer development. Consistent with this notion, *CHRNA*3 downregulation using an shRNA approach in *CHRNA*3-expressing cells leads to apoptotic defects in response to different apoptosis-inducing agents. These results argue that silencing of *CHRNA*3 expression in non-neuronal tissues (such as normal lung epithelium) may abrogate physiological cell death and promote cell survival. Transfection experiments using varying concentrations of *CHRNA*3-expression vector resulted in a dose-dependent increase in apoptotic fraction, suggesting that *CHRNA*3 gene dosage and its transcriptional control play an important role in cellular susceptibility to apoptosis. The mechanism by which *CHRNA*3 silencing contributes to cellular evasion of apoptosis appears to involve nAChR receptors whose activation induces opening of voltage-gated Ca^{2+} channels and the downstream pro-survival signalling events (8,9). This notion is supported by our results showing a significantly higher Ca^{2+} influx in *CHRNA*3-depleted cells followed by activation of MEK and Akt survival signalling pathways, inhibition of apoptosis and promotion of survival. However, it remains unclear how inactivation of *CHRNA*3 gene expression and consequent depletion of nAChR α 3 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 nAChR α 3, α 5 and β 4 subunits assemble together to form functional nAChR receptor complexes. Loss of one subunit of heteromeric nAChRs may affect activation properties of the receptors and cellular response to different receptor agonists/antagonists including nicotine, therefore higher Ca^{2+} -influx in nAChR α 3 subunit depleted cells may result from altered nicotine binding affinity and activation properties of nAChRs lacking the nAChR α 3 subunit. Alternately, these altered properties of nAChRs in nAChR α 3-depleted cells may also result from the overrepresentation of other subtype-containing nAChRs. Because nAChR α 7 receptors are the primary receptor types that mediate the proliferative effects of nicotine in the tumour cells (15) and are also considered to be the major stimulator of cancer development and progression (30,31), silencing of the nAChR α 3-encoding gene may result in overrepresentation of other nAChR subunits (notably nAChR α 7 and nAChR α 5), which may stimulate cell survival and provide a proliferation advantage to tumour cells. Based on our results we propose a model (Supplementary Figure 2) in which the *CHRNA*3 gene, apart from its classical regulatory role in neurotransmission, plays an important role in the regulation of cell survival/cell death of non-neuronal 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 non-neuronal tissue and promote tumour 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 tumours (and not in surrounding non-tumour lung tissue of same patients) the 15q25.1 locus is aberrantly methylated resulting in silencing of the *CHRNA*3 gene,

suggesting that irrespective of the known risk factor exposure, hypermethylation of *CHRNA3* gene is a common and tumour tissue-specific phenomenon. While 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 nicotinic deregulation of acetylcholine receptors in non-neuronal tissues may contribute to cancer development and progression. Our study also reinforces the interest in nicotinic acetylcholine receptors as markers for cancer detection, clinical predictors and potential chemopreventive targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. DNA Methylation analysis of the nicotine acetylcholine receptor genes clustered at the 15q25.1 locus in lung cancer

A) Schematic representation of the chromosomal arrangement of the 15q25.1 loci containing three nicotine acetylcholine receptor genes. Location of the analyzed sequence and sets of primers for each gene analyzed are indicated by grey 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)** Boxplots 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 *CHRN α 3*, *CHRN β 4* and *CHRN α 5* gene in tumour tissues (T) and blood samples

(B). **(D)** Analysis of methylation of *CHRN* genes in lung tumours and normal appearing adjacent tissues. Levels of methylation obtained for *CHRNA3*, *CHRNβ4* and *CHRNA5* genes in blood (B), lung tumours (T), and normal-appearing adjacent tissues (AT). The statistical significance for differential methylation in tissues analyzed was calculated using Newman-Keuls' test.

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 tumour samples. Representative micrographs of immunohistochemical analysis show high (upper panel) and low (lower panel) expression of nAChRα3 in the two representative lung cancer samples. Dark brown 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 tumour 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 (5-aza-2'-deoxycytidine, Aza; and RG108) for 5 days and

mRNA and methylation levels of *CHRNA3*, *CHRNβ4*, and *CHRNA5* 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 red circles represent mean expression and methylation values, respectively. The error bars represent standard error. For a given cell line, statistically significant ($P<0.05$) methylation or expression levels were calculated by comparing the treated groups with the vehicle treated group (control). Statistically significant expression or methylation values ($P<0.05$) are indicated by asterisks.

Figure 3. Restoration of expression of *CHRNA3* in cancer cells induces apoptosis

A) H1975 cells were transfected with empty vector (mock) or the vector containing the cDNA of *CHRNA3*, $\beta 4$ and $\alpha 5$ genes and expression levels of individual genes were determined by RT-PCR analysis (upper panel). GAPDH expression levels are used as a loading control. Western blot analysis of *CHRNA3* expression (lower panel). H1975 cells were transfected with empty vector (mock) or the indicated concentrations of the *CHRNA3* expressing vector, and the nAChR $\alpha 3$ 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 *CHRNA3*, $\beta 4$ and $\alpha 5$ expression vector, and the apoptotic fraction was determined 36 hours post-transfection by flow cytometry following staining

with Annexin V. The results are given as the means $\pm$ standard deviations for triplicate samples. Data are from one of two independent experiments. Statistical significance ($P < 0.05$) was calculated by comparing mock transfected samples with all other sample types in the same group, and is indicated by an asterisk. **C)** H1975 cells were mock transfected (empty vector) or transfected with the vector containing the cDNA of *CHRNA3*, and apoptotic fraction (Annexin V staining) (upper panel) and sub-G1 population (middle panel) were determined by flow cytometry. Western blot analysis of PARP cleavage after the re-expression of *CHRNA3* in H1975 cells. Full-length PARP (113 kDa) and cleaved fragments (89 kDa) are indicated. **D)** Restoration of *CHRNA3* expression is associated with activated MAPK/p38 stress signalling. H1975 cells were mock-transfected (empty vector) or transfected with indicated concentrations of the nAChR $\alpha 3$ expression vector, and 36 hours post-transfection cells were harvested and analysed by Western blotting using different antibodies.

Figure 4. Effect of the *CHRNA3* gene knockdown on nAChR signalling and apoptotic proficiency of lung cancer cells

A) H1650 and H1299 cells were stably transfected with an inducible shRNA expression vector targeting *CHRNA3* gene, and the efficiency of shRNA-mediated silencing was revealed by Western blots using anti-nAChR $\alpha 3$ antibody. To silence the *CHRNA3* gene, expression of shRNA was induced with 1 μ M doxycycline (+Dox) for 48 hours. **B)** Analysis of nicotine-induced Ca^{2+} influx in H1299 cells expressing different levels of nAChR $\alpha 3$. H1299 cells were grown in the presence (+Dox) or absence (-Dox) of doxycycline, and following addition of 10 mM nicotine (time point, 0 sec), the imaging of intracellular Ca^{2+} was performed using the Ca^{2+} indicator fluo-4 (10 μ M) and confocal laser microscopy (upper panel). RGFP expression is under the control of the same doxycycline-inducible promoter as the $\alpha 3$ -shRNA and thus is used as a reporter to shRNA expression. Plot comparing the quantified fluorescent intensity changes in *CHRNA3*-depleted (+Dox) and *CHRNA3*-expressing (-Dox) cells (lower panel). **C)** Knockdown of *CHRNA3* expression

triggers activation of the Akt-signalling pathway. H1299 cells were treated with nicotine (10 mM) for 48 hours following prior incubation in the presence (+Dox) or absence (-Dox) of doxycycline for 48 hours. 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 *CHRNA3* gene impairs cell death response. H1650 and H1299 cells stably transfected with inducible *CHRNA3* shRNA were incubated in the presence or absence of doxycycline for 24 hours and treated with etoposide, cyclohexamide or dexamethasone (100µM) for a further 24 hours. Cells were then stained with Annexin V/propidium iodide, and apoptotic fraction was analysed by FACS. Shown are mean values (+/-SD) from three different experiments. Statistical significance was calculated using Student's unpaired 't' test ($P < 0.05$; two tailed) and significant values are indicated by an asterisk.

Table 1

Frequency of CpG hypermethylation in lung tumours

Gene	Methylation levels Maximum/Minimum/Average	Methylation level cut-off [^]	Hypermethylated tumor samples [*] (%)
<i>CHRNA3</i>	64.83% / 0% / 21.14%	22.67%	52/142 (36.6%)
<i>CHRNβ4</i>	51.14% / 0% / 13.36%	15.03%	30/124 (24.2%)
<i>CHRNA5</i>	7.51% / 0% / 1.57%	5.72%	7/103 (6.8%)

[^] The 90% percentile value for surrounding non-tumour lung tissue samples.

^{*} Samples with methylation levels above the quantile representing the upper 90% of methylation in surrounding non-tumour lung tissue samples.