Acetylcholine receptor pathway in lung cancer: New twists to an old story

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Abstract

Genome-wide association studies revealed that allelic variation in the α5-α3-β4 nicotine acetylcholine receptor (nAChR) cluster on chromosome 15q24-15q25.1 was associated with lung cancer risk. nAChRs are membrane ligand-gated cation channels whose activation is triggered by the binding of the endogenous neurotransmitter acetylcholine (ACh) or other biologic compounds including nicotine. nAChRs have been found on lung cancer cells, underscoring the idea that the non-neuronal nAChR signaling pathway has considerable implications for lung cancer. Several studies involving the design of nAChR antagonists with improved selectivity might identify novel strategies for the treatment of lung cancer.

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INTRODUCTION

Lung cancer is a multifactorial disease, resulting from a combination of genetic, environmental, and psychological factors. The evidence that genetic factors influence lung cancer risk has been demonstrated by many studies, tracing back to the landmark study in 1963[1]. This study demonstrated a 2.5-fold higher risk in smoking relatives of lung cancer cases compared with smoking relatives of controls[1], fully suggesting the importance of genetic factors in lung cancer risk.

More recently, some large genome-wide association (GWA) studies identified an association between single-
with the arrival of genotyping and genomic profiling Lung cancer is a heterogeneous and challenging disease to understand, and the molecular mechanisms of the cholinergic pathway, including the nAChR gene cluster, the roles of nicotine receptors, and the exogenous compound nicotine activated by endogenous agonist acetylcholine (ACh) and superfamily of ligand-gated ion channels (CHRN) genes, are members of the Cys-loop superfamily of ligand-gated ion channels, which are activated by endogenous agonist acetylcholine (ACh) and the exogenous compound nicotine and nitrosamines, potential lung carcinogens in cigarette smoke and foods.

CHRN genes are expressed in both neuronal and non-neuronal tissues, suggesting that nAChRs may play an important role in processes other than synaptic transmission. Indeed, apart from the 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. A decreased survival correlation with lung cancer may be explained by the effects of the nAChRs pathway through CHRNA proteins on tumor cell proliferation, apoptosis, epithelial-mesenchymal transition, and proinvasive and angiogenic phenotypes. This review will provide an overview for better understanding the genetic risk factors for lung cancer in the nAChR gene cluster, the roles of nicotine receptors, and the molecular mechanisms of the cholinergic pathways to lead to more opportunities for intervention and prevention of lung cancer.

**GENETIC RISK FACTORS IN THE NACHR GENE CLUSTER FOR LUNG CANCER**

**GWA studies for lung cancer**

Lung cancer is a heterogeneous and challenging disease to treat. With the arrival of genotyping and genomic profiling, management of non-small-cell lung cancer (NSCLC) has made several advances with the understanding of activating mutations in epidermal growth factor receptor (EGFR), fusion genes involving anaplastic lymphoma kinase (ALK), rearrangements in ROS-1. The next era of personalized treatment will involve a comprehensive genomic characterization of lung cancer. One important task in personalized medicine is to predict disease risk based on a person's genome, e.g., on a large number of SNPs. GWA studies make SNP and phenotype data available to researchers.

Notably, the 15q24-15q25.1 region (CHRNA5/CHRNB4) has been identified as a lung cancer risk spot by several GWA studies. Of one GWA study in the International Agency for Research on Cancer (IARC), there is 14% increased lung cancer susceptibility associated with nAChR cluster variation irrespective of smoking status. The same GWA result was identified by the other studies from the MD Anderson Cancer Center, United States, and deCODE Genetics, Iceland, respectively. Importantly, a non-synonymous variant rs16969968 of CHRNA5, inducing an amino acid substitution (D398N) in the second intracellular loop of the protein, can increase lung cancer risk by 30%. The SNP rs8023462 in CHRNA3/CHRNB4 intergenic region, interfering CHRNA3/CHRNB4 gene expression by interacting with GATA transcription factors, was the first functional evidence for association with lung cancer risk.

To better understand the GWA studies for the nAChR gene cluster and lung cancer risk, we further investigate a catalog of all existing published GWA study data updated to December 20, 2013 from National Human Genome Research Institute (NHGRI) (http://www.genome.gov/), using the subject terms (CHRNA OR nAChR OR 15q25) AND (lung cancer OR lung adenocarcinoma OR 15q21.3) OR (lung cancer OR lung adenocarcinoma OR lung cancer-asbestos exposure interaction). Twenty potentially relevant articles were produced according to the first primary screening strategy, of which 7 GWA studies met the subject terms of genetic variants and the financial constraints on the numbers of variants, it is stringent to perform meta-analyses of existing genetic data on the nAChR gene cluster to better understand disease loci harboring common variants associated with lung cancer risk. A total of 12 qualified articles between 2008 and 2011 screened from 40 potentially relevant articles were selected, including 16 studies with 9 in Caucasians, 4 in East Asians, 2 in African-Americans, and 1 in mixed (Caucasian, African-American and Hispanic) populations. CHRNA3 gene

Figure 1 Flow diagram of search strategy and study selection.
rs1051730-A allele, compared with G allele, is associated with a 36% higher risk for lung cancer (95%CI, 1.27-1.46; \( P < 0.0005 \))\[35\]. Surprisingly subgroup analyses suggested that rs1051730 allele might be the factor for lung cancer susceptibility in Caucasians (OR = 1.32; 95%CI, 1.25-1.44; \( P < 0.0005 \)), but not in East-Asians (OR = 1.51; 95%CI, 0.76-3.00; \( P = 0.237 \))\[36\]. Allele frequency of rs1051730 in Asians was lower than that in Caucasians. For rs1051730-A allele, compared with G allele, is associated with susceptibility in Caucasians (OR = 1.32; 95%CI, 1.25-1.44; \( P < 0.0005 \)), but not in East-Asians (OR = 1.51; 95%CI, 1.15-1.47)\[37\]. Small hairpin RNA-mediated depletion of CHRNA3 in CHRNA3-expressing lung cancer cells led to resistance to apoptosis-inducing agents, underscoring the importance of epigenetic silencing of the CHRNA3 gene in human cancer\[38\]. Silencing of the CHRNA3-encoding gene may result in over-representation of other nAChR subunits, notably CHRNA7 and CHRNA5\[39\], which may stimulate cell survival and proliferation advantage to tumor cells\[40,41\]. In contrast to CHRNA3 hypermethylation, elevated CHRNA4-methylation, insufficient to induce significant silencing of the gene, failed to inhibit gene expression. \( P < 0.0005 \).

### Table 1  Qualified genome-wide association studies at the 15q25.1 region on the risk of lung cancer

| Date   | PubMed ID  | Initial sample size | Replication sample size | Chromosome position | Mapped gene | SNP | Context | P value | OR (95%CI)   | Platform |
|--------|-------------|---------------------|-------------------------|---------------------|-------------|-----|---------|---------|-------------|----------|
| 8/4/2009 | 19654303    | Broderick et al\[42\] | 1952 European ancestry cases, 1438 European ancestry controls | 15q25.1 | AGPHD1 | rs8034191 | intron 5 | 7880023 | 3.00E-26  | 1.29 (1.23-1.35) | Illumina |
| 11/2/2008 | 18978790    | McKay et al\[43\]   | 2971 cases, 3746 controls | 15q25.1 | CHRNA3 | rs1051730 | cds-synon | 7894339 | 1.00E-15  | 1.35 (1.25-1.45) | Illumina |
| 11/2/2008 | 18978787    | Wang et al\[44\]    | 1952 cases, 1438 controls | 15q25.1 | CHRNA3 | rs1051730 | cds-synon | 7890032 | 8.00E-12  | NR | Illumina |
| 9/17/2008 | 18780872    | Liu et al\[45\]     | 194 cases, 219 controls | 15q25.1 | CHRNA3 | rs1051730 | intron 1 | 7880023 | 1.00E-8   | 1.38 (1.17-1.64) | Illumina |
| 4/3/2008  | 18385676    | Amos et al\[46\]    | 1514 cases, 1137 controls | 15q25.1 | CHRNA3 | rs1051730 | intron 3 | 7880023 | 3.00E-18  | 1.30 (1.15-1.47) | Illumina |
| 4/3/2008  | 18385738    | Hung et al\[47\]    | 1926 cases, 2522 controls | 15q25.1 | CHRNA3 | rs1051730 | intron 8 | 7880023 | 5.00E-20  | 1.30 (1.23-1.37) | Illumina |
| 10/15/2009 | 19836008   | Landi et al\[48\]   | 5799 European descent cases, 5848 European descent controls | 15q25.1 | CHRNA3 | rs1051730 | intron 1.00E-26  | 1.31 (1.27-1.36) | Illumina |

### Interplay of smoking behaviors, nAChR cluster and lung cancer risk

Evidence that the nAChR cluster on 15q25 locus is associated with smoking status, nicotine dependence and the risk of lung cancer is inconsistent in different populations. The region of the nAChR cluster has been confirmed to be associated with a number of smoking-related traits, including nicotine dependence, cigarettes smoked per day, and heavy smoking, in some lung cancer GWA studies\[42,43\], and in some genome-wide meta-analyses in Caucasian populations\[44,45\]. For example, the Caucasian population with variant rs1051730 SNP in the nAChR cluster was related with lung cancer risk and...
nicotine dependence, approximately smoking one and two more cigarette per day than those without variant rs1051730 SNP[48]. Another study demonstrates further that association signals in the nAChR cluster affecting early smoking behaviors may have disparity with those contributing the mature nicotine dependence[49].

Surprisingly, a Japanese case-control study[36] reported associations between the selected SNPs in the nAChR cluster and risk of lung cancer and found that associations among never and ever smokers were similar. The association was consistent among non-smokers and smokers in our study[19]. These studies might argue for a role of the nAChR cluster in lung cancer that is independent of smoking behavior in Asians.

These findings in different populations suggest a role for racial differences in the association between smoking behaviors, nAChR cluster, and risk of lung cancer. Reasons underlining the racial difference in the genotype with smoking associations are unclear. This discrepancy may be due to differences in genetic and environmental backgrounds. Alternatively, other factors that have not been taken into account, such as food intake and passive smoking, differentiate the mode of contribution of the nAChR cluster in non-smokers.

**STRUCTURE AND FUNCTION OF NACHRS**

The regulation of acetylcholine receptor pathways has been established from the primitive organisms, irrespective of neurons. There exist two types of AchRs, nAChRs and Muscarinic ACh receptors (mAChRs) of cholinergic signaling, nAChRs comprise five subunits, including ten α subunits (α1-α10), four β subunits (β1-β4), one δ, and one ε or γ subunit, which form hetero- or homo-pentamers enclosing a central ion channel. The nAChR subunit composition in turn further regulates the function and pharmacology of nAChRs[16,17].

**An autocrine or a paracrine acetylcholine receptor pathway for nAChRs**

Acetylcholine receptor signaling in non-neuronal cells is comparable to acetylcholine receptor neurotransmission[46]. Both nAChR families are expressed in cancer cells[48], and both NSCLC and small-cell-lung cancer (SCLC) cell lines can synthesize and release Ach[49]. The widespread synthesis of Ach beyond the nervous system has changed the paradigm of Ach acting merely as a neurotransmitter, and it may also act as an autocrine or a paracrine messenger able to interact with nAChRs. For example, non-neuronal Ach is released from the living NSCLC or SCLC cells and binds to nAChRs of its source and neighbouring cells to mediate autocrine and paracrine regulatory loops, prolonging cell survival with subsequent cell proliferation through mitogen-activated protein kinase (MAPK) pathway[48-50] or with increase of vascular endothelial growth factor (VEGF) stimulating neoangiogenesis[19]. All these effects can be blocked at the level of the nAChRs[48,51,52]. This novel paradigm necessitates the opportunity of marker-guided lung cancer intervention and prevention strategies, making balance between nAChR-mediated stimulation and inhibition[16,53].

**Metabolism of Ach**

Ordinarily Ach is regarded as a classical neurotransmitter. Ach is synthesized intracellularly by the enzyme choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A (AcCoA) before being released into the extracellular space to act on synaptic-adjacent cells. In contrast, the enzyme acetylcholinesterase (AChE) can rapidly clear the extracellular Ach pool into its inactive metabolites choline and acetate. This signaling system is targeted by various biological modulators which can inhibit Ach release or AchE activity[94]. ChAT is strongly up-regulated, whereas AChE is down-regulated in squamous cell carcinoma (SqCC)[54], which increases levels of Ach, providing endogenous proliferative stimuli to nAChRs.

**Classical ionic channel activity of nAChRs**

All nAChRs allow the influx of different cations (Na+, K+, Ca2+), and the α7nAChR is selective for Ca2+. Binding of nicotine to α7nAChR can cause Ca2+ influx into lung cancer cells and the subsequent membrane depolarization activates voltage-gated Ca2+ channels, which activates the MAPK pathway[48]. Subsequently, MAPK activates complexes of the transcription factor NF-kB that induce entry into S phase to promote cancer cell proliferation[48]. Moreover, tobacco-specific nitrosamine 4-(methylnitosamino)-1-(3-pyridyl)-1-butaneone (NNK) has been found to promote Ca2+ influx thought binding to nAChRs, activating both protein kinase C (PKC) and the MAPK pathway to stimulate the proliferation of SCLC cells[49].

**Cell proliferation mediated by nAChRs**

The role of nAChRs in the growth regulation of cancer was first identified in 1989[13], and reinforced further by the ability of generalized nAChR antagonists (e.g., hexamethonium and mecamylamine) to reverse the proliferative effects of nicotine. Nowadays, the mechanism of nicotine-induced tumor cell proliferation and associated angiogenesis is an active area of research. In particular, α7nAChR has been implicated in mediating the proliferative effects of nicotine, and α7nAChR antagonist a bungarotoxin (α-Btx) or methyllycaconitine can attenuate the proliferative effects of nicotine in NSCLC and SCLC cells[16,52]. These findings have been confirmed by the transfection of small interfering RNA targeting α7nAChR in lung cells[52]. This point implies that α7nAChR is being considered a target for cancer therapy[48], marking a new chapter in lung cancer research and the feasibility of using nAChRs as a viable molecular target for cancer therapy.

**Cross-talk of nAChRs with the other signaling pathways**

Beyond the above channel activity, nAChRs can be involved in other intracellular events involving various
downstream nAChR-mediated signaling pathways, including Ca\textsuperscript{2+}/calmodulin, PKC\textsuperscript{[49]}, MAPK\textsuperscript{[48-50]}, and VEGF\textsuperscript{[19]}. It has been reported that EGFR was found to be on the list of high α6β3 tumors, and nAChR may trigger the MAPK pathway in which EGFR was involved, leading to promotion of cell growth and proliferation. Thus, cross-talk between signaling downstream of EGFR and nAChR activation via the MAPK pathway may together promote carcinogenesis\textsuperscript{[50]}. The other findings suggest a strong connection between growth factor mediated angiogenic pathway and the cholinergic angiogenic pathway\textsuperscript{[19,55]}. For instance, the endothelial cell (EC) migration induced by basic fibroblast growth factor (bFGF) and VEGF can be inhibited by blocking the nAChRs with α-BTx\textsuperscript{[19,55]}. Figure 2 simply summarizes the above mentioned nAChRs pathway in lung cancer.

Figure 2  nAChRs pathway in lung cancer. (1) Ach is synthesized intracellularly by the enzyme choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A (AcCoA) before being released into the extracellular space. This signaling system is targeted by various biological modulators which can inhibit Ach release or ChAT activity. ChAT is strongly up-regulated in squamous cell carcinoma (SqCC), which increases levels of Ach, providing endogenous proliferative stimuli to nAChRs; (2) All nAChRs allow the influx of different cations (Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}), and the α7nAChR is selective for Ca\textsuperscript{2+}. Binding of nicotine to α7nAChR can cause Ca\textsuperscript{2+} influx into lung cancer cells and the subsequent membrane depolarization activates voltage-gated Ca\textsuperscript{2+} channels, which activates the MAPK pathway to promote cancer cell proliferation; (3) Hypermethylation of 15q25.1 locus and epigenetic silencing of the CHRNA3 gene may result in under-representation of α3 containing nAChR-subtypes, which in turn leads to over-representation of α7 and α5 containing nAChRs on the cell surface, further increasing Ca\textsuperscript{2+} influx mediated activation of the MAPK signaling cascade. As a result, cells with the complete or partial absence of the CHRNA3 subunit containing nAChRs may display defective cell death response; (4) α7nAChR antagonist abungarotoxin (α-BTx) can attenuate the proliferative effects of nicotine in NSCLC and SCLC cells; and (5) The other findings suggest a strong connection between growth factor mediated VEGF pathway and the cholinergic angiogenic pathway. The endothelial cell (EC) migration induced by basic fibroblast growth factor (bFGF) and VEGF can be inhibited by blocking the nAChRs with α-BTx.
NACHRS RESEARCH IN SOME SPECIAL LUNG CANCERS

Lung cancer in non-smokers

The carcinogenic nitrosamine N-nitrosodiethylamine (DeN) was identified recently to have the similar structure with ACh, acting as a high-affinity ligand for homo- and hetero-meric nAChRs in lung cancer cells[36]. Considering the fact that DeN is contained in numerous foods and drinks[37], such agents may lead to non-tobacco-related modulations of nAChRs. In addition, different nAChR subunit gene expression patterns were found between NSCLCs in smokers and non-smokers, and higher nAChR α6β3 expression was associated with NSCLC tumors from non-smokers, as compared with those from smokers[38].

Lung cancer in female patients

Neurotransmitter γ-aminobutyric acid (GABA) is recognized as the most important inhibitory neurotransmitter in the brain, but it also acts as a tumor suppressor for pulmonary adenocarcinoma (PAC)[39] and carcinomas of the pancreas[40], breast[41] and colon[42]. As α4β2nAChR regulates the release of GABA, desensitization of this receptor may therefore lead to GABA deficiency[43]. Moreover, it has been shown that mRNA levels of the α4nAChRs were significantly lower in PAC tissues than in normal lung tissue or other types of NSCLCs[8]. Considering that oestrogen and phyto-oestrogens were reported to desensitize α4β2nAChR[44], the predominance of PAC in women may therefore at least in part be the result of impaired α4β2nAChR function. Recent reports of clinical trials of the nAChR antagonist α4β2b antagonist, which targets α4β2 receptors in the brain, have shown its clinical efficacy in smoking cessation[45]. It is possible that similar nAChR antagonists could block the effect on lung tumors, including lung cancer in women.

Lung cancer in pulmonary neuroendocrine cells

Pulmonary neuroendocrine cells (PNeCs) and SCLC can express high levels of α7nAChR[46]. α7nAChR is the central regulator of proliferation, apoptosis and migration in SCLC cells through stimulating the release of some autocrine growth factors, including serotonin and neu- ropeptides[47,48]. α7nAChR antagonist α-BTts can attenuate the proliferative effects of nicotine in SCLC cells[49,50].

SURVIVAL STUDIES FOR NACHRS

nAChRs are associated with resistance to gemcitabine, cisplatin and paclitaxel in NSCLC cell lines. Our research is the first study to investigate whether or not a genetic variant in the 15q25 region has a prognostic effect on the survival outcome of patients with lung cancer[35]. The patients with CHRNA3 gene rs3743073G > T allele showed a higher risk of lung cancer and worst survival in Chinese Han patients with advanced stage NSCLC[35]. A Caucasian study showed that CHRNA3 (rs1051730) genotyping can improve customized chemotherapy based on tumor assessment of excision repair cross-complementing 1 (ERCC1) mRNA in stage IV NSCLC patients with a performance status of 0 [clinicaltrials.gov identifier: NCT00174629][50]. A further Korean survival study demonstrated that a functional SNP, rs6495309CT > T, in the promoter of the CHRNA3 gene, was the prognostic factor for resected early stage NSCLC[60]. Compared with rs6495309 CC genotype, the patients in the studied Korean cohort with rs6495309 CT/TT genotype had a better 5-yr OS by 5% and better 5-yr DFS by 7%[61].

NACHRS AS BIOMARKERS FOR LUNG CANCER - THE FUTURE PERSPECTIVE?

The use of nAChR antagonists that block the receptors still has some issues, because these receptors regulate many vital cell and organ functions, and deficiency or impairment of nAChR signaling will lead to overproduction of cytokines in some tissues and enhance tissue damage. Carefully designed animal studies are essential to investigate the potential side effects of nAChR antagonists on the brain, central nervous system, immune cells and muscle cells, which express high levels of nicotinic receptors.

In summary, strategies for marker-guided lung cancer intervention that targets nAChRs seem promising. nAChR antagonists could be potentially used in combination with established chemotherapeutic drugs to enhance the therapeutic response to chemotherapy. Future studies involving the design of nAChR antagonists with improved selectivity might trigger novel strategies for the intervention and prevention of lung cancer.

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