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Review

Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer

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ABSTRACT

Despite the known adverse health effects associated with tobacco use, over 45 million adults in the United States smoke. Cigarette smoking is the major etiologic factor associated with lung cancer. Cigarettes contain thousands of toxic chemicals, many of which are carcinogenic. Nicotine contributes directly to lung carcinogenesis through the activation of nicotinic acetylcholine receptors (nAChRs). nAChRs are ligand-gated ion channels, expressed in both normal and lung cancer cells, which mediate the proliferative, pro-survival, angiogenic, and metastatic effects of nicotine and its nitrosamine derivatives. The underlying molecular mechanisms involve increases in intracellular calcium levels and activation of cancer signal transduction pathways. In addition, acetylcholine (ACh) acts as an autocrine or paracrine growth factor in lung cancer. Other neurotransmitters and neuropeptides also activate similar growth loops. Recent genetic studies further support a role for nAChRs in the development of lung cancer. Several nAChR antagonists have been shown to inhibit lung cancer growth, suggesting that nAChRs may serve as valuable targets for biomarker-guided lung cancer interventions.

Contents

1. Lung cancer facts and figures

Lung cancer is one of the most common types of cancer, accounting for approximately 15% of all cancer cases worldwide [1]. In the United States, the incidence rate for lung cancer is surpassed only by that for prostate cancer in males and breast cancer in females [2]. However, lung cancer remains the leading cause of cancer-related mortality around the world, resulting in more than 1 million deaths per year. In the United States, lung cancer poses a substantial economic burden on the healthcare system, averaging $6250 monthly in total healthcare cost per patient [3]. Annual productivity loss due to the disease is approximately $23 billion for males and $14 billion for females [4].

Tobacco use is the main risk factor associated with lung cancer, with lung cancer prevalence over time reflecting rates of tobacco consumption *per capita* [5]. In developed countries, smoking is estimated to cause more than 80% of lung cancer cases [1]. In the United States, 24% of male smokers are expected to develop lung cancer in their lifetime, with a 5- to 10-fold increase in risk compared to non-smokers. Overall prognosis for the disease remains dismal, with 5-year survival rates ranging from 6% to 14% for males and 7% to 18% for females [6].

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Lung cancer has traditionally been classified into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), based on histological characteristics. SCLC is the most aggressive type of lung cancer and has the poorest survival rate [7]. SCLC arises from pulmonary neuroendocrine cells and can be grouped with other tumors that develop from these precursor cells, namely, typical carcinoid tumors, atypical carcinoid tumors, and large cell neuroendocrine carcinomas. Pulmonary neuroendocrine cells are either found in isolation throughout the lung and airways or in small groups called neuroepithelial bodies, typically found at bifurcations of small airways. Neuroepithelial bodies synthesize neurotransmitters and neuropeptides as well as growth factors and vasoactive substances [8]. They play a trophic role in lung development and function as oxygen sensors and possibly chemo- and mechanoreceptors [9–11].

NSCLC can be subdivided into adenocarcinomas, squamous cell, and large cell lung carcinomas. Adenocarcinomas can be further classified as acinar, papillary, bronchioalveolar, solid adenocarcinoma with mucin production, and mixed subtypes [6]. Adenocarcinomas develop from small airway epithelial cells and alveolar type II cells while squamous cell carcinomas are derived from large airway epithelial cells. Biomarker-guided therapies against NSCLC target specific cell types and subtypes [6,12].

2. nAChR expression in lung cells

Though traditionally labeled "neuronal," it has become evident that neuronal nAChRs are expressed in numerous cell types and tissues including endothelial cells, gastrointestinal tissue, glia, immune cells, keratinocytes, urinary bladder tissue, reproductive organs, and respiratory tissue [13–20]. Neuronal nAChRs are made up of homomeric or heteromeric combinations of α and β subunits, which include α2–α10 and β2–β4 [21–23]. Five subunits co-assemble to form a cation-selective channel. nAChR subtypes can be divided into two main classes: (1) α-bungarotoxin (αBgtx)-sensitive subtypes such as α7, α8, α7/α8, α9, and/or α10 and (2) heteromeric αBgtx-insensitive subtypes including combinations of α2-α6 with β2–β4 [24]. α7 and α4β2-containing nAChRs are the most abundant subtypes expressed in the central nervous system while α3β4-containing nAChRs are the predominant subtypes expressed in the peripheral nervous system [23,25]. While the specific combinations of subunits in native nAChRs have not been completely elucidated, it is clear that a huge diversity of receptor subtypes may exist, each subtype having distinct pharmacological and biophysical properties [25].

In the lung and airways, nAChR subunit transcripts have been detected using RT-PCR, qRT-PCR, and in situ hybridization and have been shown to be expressed at varying levels in normal and malignant cells [17,26–29]. Protein expression of a variety of these nAChR subunits has also been investigated using Western blot analysis while assembly of functional nAChRs on the cell surface has been determined using radioligand binding assays and patch clamping [17,30–32]. The near ubiquitous expression of nAChRs in lung cells underscores the need to elucidate their functional relevance in normal physiology and in disease states.

3. ACh autocrine/paracrine loop

ACh is a phylogenetically ancient molecule that serves as the endogenous ligand for nAChRs [14]. In the central and peripheral nervous systems, ACh plays a role in synaptic transmission, nociception, learning and memory, sleep cycle regulation, and neuroendocrine function [33,34]. Outside the nervous system, ACh synthesis was first described in placental tissue [35]. Since then, ACh has been found in a variety of non-neuronal cells and tissues [14,36–38].

In normal respiratory tissue, ACh is secreted by large airway epithelia, small airway epithelia, and pulmonary neuroendocrine cells [39,40]. The ACh signaling machinery in these cells requires many of the cellular components that are found in neurons [36]. In particular, ACh signaling in lung cells is also mediated by nAChRs [29,41]. Additionally, ACh is synthesized by the enzyme choline acetyltransferase (ChAT) and is degraded by the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [36,42]. One key difference is that neurons require the high affinity choline transporter (CHT1) for ACh synthesis whereas not all lung cells express CHT1 [36]. Instead, lung cells use choline-transporter like proteins (CTLs) [43]. Another distinguishing characteristic is that neurons package ACh into vesicles via the vesicular ACh transporter (VAChT) whereas in lung cells, ACh secretion does not necessarily require VAChT [36].

Lung cancers secrete ACh and utilize the same ACh signaling components as normal lung cells [42,44]. In these cells, ACh acts as an autocrine or paracrine growth factor that activates a feedback loop leading to cell proliferation (Fig. 1). The non-selective nAChR antagonist mecamylamine inhibits lung cancer growth, suggesting that the ACh machinery can be exploited in the rationale design of therapeutics against lung cancer [36]. Possible pathway points that can be targeted include ACh synthesis, ACh secretion, receptor activation, choline uptake, downstream pathways, and interactions with proteins such as L6d neurotoxin 1 (lynx1) and secreted mammalian Ly6/UPAR-related proteins 1 and 2 (slurp-1/2) [36,45–48].

In addition to ACh, several other neurotransmitters and neuropeptides act as autocrine or paracrine growth factors in lung cancer including bombesin/gastrin-releasing peptide, bradykinin, catecholamines, cholecystokinin, galanin, litorin, neuromedin, neurotensin, ranatensin, serotonin, and vasopressin [49]. Bombesin and serotonin release has been shown to be dependent on protein kinase C (PKC) activation [50]. Growth factors such as the epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) also act as autocrine or paracrine mitogenic signals in lung cancer [51,52].

4. Receptor-mediated activation of cancer signaling pathways

Through the years, it has become apparent that nAChRs in lung cells act as central mediators in the activation of cancer signaling pathways [51]. As early as 1989, Schuller [53] showed that nicotine stimulates proliferation of a neuroendocrine lung cancer cell line, an effect that could be abolished by nAChR antagonists. Shortly thereafter, John Minna's group showed that lung cancer cells do express nAChRs and that nicotine inhibits apoptosis in these cells [32]. Receptor-mediated effects of nicotine were subsequently recapitulated by other nAChR agonists such as cytisine and the tobacco nitrosamines 4-(methlynitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN).

Signaling begins with the binding of agonists to nAChRs, causing a conformational change that leads to the opening of the channel and the influx of cations such as Na^+ and Ca^{2+} and the efflux of K^+ (Fig. 1). The resulting membrane depolarization opens the gates of voltage-operated calcium channels (VOCCs), leading to additional flow of Ca^{2+} [51]. Calcium influx triggers the secretion of mitogenic factors and activates signaling cascades involved in cell proliferation, apoptotic inhibition, migration, and angiogenesis [50,51,54].

4.1. Cell proliferation

Nicotine exposure alone does not appear to initiate lung cancer, though nicotine exposure combined with hyperoxia can induce lung tumors in hamsters, suggesting that chronic nAChR activation

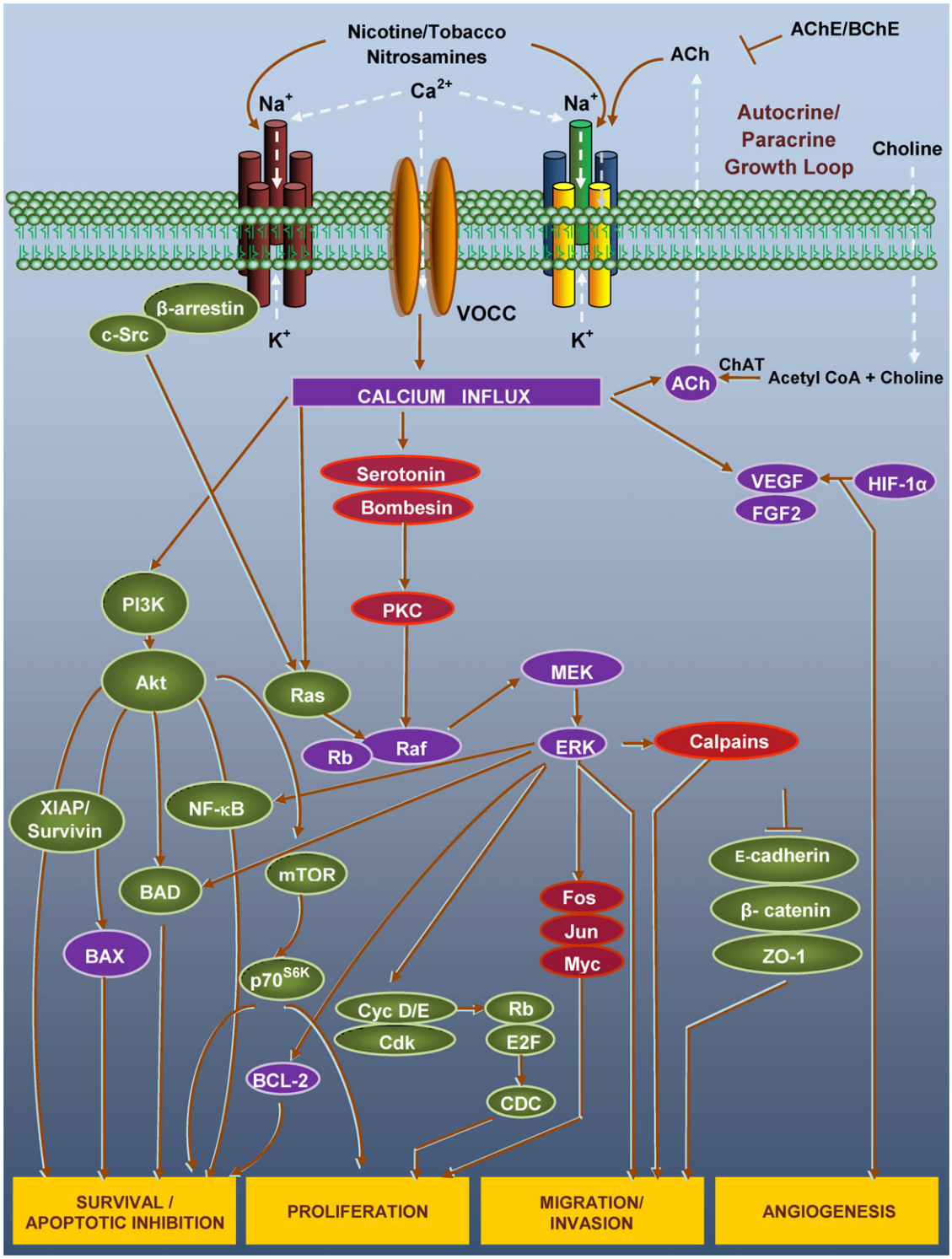

Fig. 1. Model of nAChR-mediated cancer signaling pathways. nAChR agonists such as ACh, nicotine, and tobacco nitrosamines bind to and activate homomeric (red) or heteromeric (multicolored) nAChRs. nAChR activation leads to opening of the channel and the flow of Na^+, K^+, and Ca^{2+} ions down their electrochemical gradients. Subsequent membrane depolarization opens voltage-operated calcium channels and further increases intracellular calcium levels. Calcium influx – whether in series, in parallel, or in combination with other receptor-mediated mechanisms – activates downstream signal transduction pathways leading to cancer cell survival, proliferation, migration/invasion, and angiogenesis. Calcium influx also triggers the secretion of neurotransmitters and neuropeptides such as bombesin and serotonin that act as autocrine or paracrine growth factors in lung cancer. Pathway components in red indicate those identified in SCLC and its precursor cells; those in green have been identified in NSCLC and its precursor cells; and those in purple have been identified in both types of cancers and corresponding cells of origin. Abbreviations: Acetyl-CoA – acetyl coenzyme A; ACh – acetylcholine; AChE – acetylcholinesterase; BAD – Bcl-2-associated death promoter; BAX – Bcl-2-associated X protein; Bcl-2 – B cell lymphoma 2; BChE – butyrylcholinesterase; CDC – cell division cycle; Cdk – cyclin dependent kinase; ChAT – choline acetyltransferase; Cyc D/E – cyclin D or E; ERK – extracellular signal-regulated kinase; FGF – fibroblast growth factor 2; HIF-1α – hypoxia-inducible factor-1 alpha; MAPK – mitogen activated protein kinase; MEK – MAPK/ERK kinase; nAChR – nicotinic acetylcholine receptor; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K – phosphatidylinositol 3-kinase; PKC – protein kinase C; Rb – retinoblastoma; VEGF – vascular endothelial growth factor; VOCC – voltage-operated calcium channels; XIAP – X-linked inhibitor of apoptosis protein; ZO-1 – zonula occludens 1.

along with impaired oxygenation can initiate tumorigenesis [55]. Nicotine, more likely, acts to promote tumorigenesis after it has been initiated. In NSCLC and their normal cells of origin, nicotine triggers the release of EGF, leading to the binding of EGF to its cognate receptor, EGFR, and activation of the Ras-Raf-ERK cascade, a signal transduction pathway that leads to cell proliferation [56,57]. Activation of the Ras-Raf-ERK pathway can be abrogated by the α7 nAChR antagonists, α-BTx, and α-cobratoxin (α-Ctx) [58].

β-arrestin signaling has also been implicated in nicotine-induced cell proliferation [59]. The binding of β-arrestin to α7 nAChRs activates Src and Raf pathways and causes subsequent binding of Raf to the retinoblastoma protein (Rb). Rb inactivation and association with cyclins D and E (cyc D/E) lead to the activation of E2F-regulated transcription and entry into S-phase. Signaling through this pathway can be inhibited by the general nAChR antagonist, hexamethonium, as well as by α-BTx and methyllycaconitine (MLA), another α7 nAChR antagonist [60].

Nicotine also induces fibronectin production, which activates the extracellular signal-regulated kinase (ERK), the phosphatidylinositol 3-kinase (PI3-K), and the mammalian target of rapamycin (mTOR), leading to cell proliferation and survival [61]. In addition, nicotine stimulates the expression of the peroxisome proliferator-activated receptor (PPAR-β/δ)aneffect that can be blocked by α-BTx, α7 small interfering RNAs (siRNAs), and PI3-K and mTOR inhibitors [62].

Carcinogenic nitrosamines such as NNK and NNN also promote cell proliferation by activating distinct signaling pathways. NNK has a higher affinity for α7 nAChRs while NNN has a higher affinity for heteromeric nAChRs [63]. In SCLC, NNK evokes calcium influx and activates the Raf-MAPK pathway activation, resulting in the phosphorylation of c-myc [64,65]. Tobacco nitrosamines can therefore promote lung cancer through direct genotoxic effects or via nAChR-mediated mechanisms [66,67].

4.2. Survival pathways

Nicotine confers resistance to the apoptotic effects of chemotherapeutic drugs, opioids, oxidative stress, and UV radiation [68–70]. The pro-survival effects of nicotine appear to involve the PI3-K-Akt pathway [71]. Nicotine causes site-specific phosphorylation of Akt and downstream substrates such as GSK-3, $p70^{S6K}$, 4EBP-1, and FKHR [72]. Akt is a known physiological kinase of Bcl-2 family members. Consistent with this, nicotine exposure activates Bcl-2, a key anti-apoptotic molecule while inactivating the pro-apoptotic proteins, Bad and Bax [68,73,71]. Similarly, NNK inhibits apoptosis by activating Bcl-2, an effect that can be blocked by inhibitors of PKC and ERK1/2 and by c-myc silencing [74]. Akt activation also leads to the upregulation of the X-linked inhibitor of apoptosis protein (XIAP) and survivin, both inhibitors of apoptosis [75]. Other pathways implicated in nAChR-mediated cell survival involve β-adrenergic receptor activation as well as NF-κB activation [76,77].

4.3. Tumor angiogenesis

Tumors can use simple diffusion to feed on oxygen and nutrients although past a certain point they require communication with vascular endothelial cells and formation of new blood vessels for further growth [78]. Angiogenesis, the process of forming new blood vessels, involves (1) activation of endothelial cells by angiogenic stimuli such as hypoxia and cytokine release, (2) degradation of the basement membrane by matrix metalloproteinases, and (3) proliferation and migration of endothelial cells towards the angiogenic stimuli, via a vascular growth factor (VEGF)-dependent mechanism [78–81]. Vascular endothelial cells express nAChRs as well as other components of the ACh signaling machinery [16,82]. In these cells, ACh also acts in an autocrine or paracrine fashion and modulates vascularization and remodeling [83,84]. Nicotine and its metabolite cotinine can induce endothelial cell tube migration by stimulating VEGF expression in lung cancer cells, an effect that can be reduced by α-BTx, MLA, and mecamylamine [84–87]. Inhibition of the MAPK and PI3-K pathways prevents nicotine-induced neovascularization [84]. Nicotine, in combination with estradiol, also enhances growth of lung cancer xenografts via increased cell proliferation, VEGF secretion, and angiogenesis [88]. Finally, nicotine stimulates accumulation of hypoxia-inducible factor-1a (HIF-1α), a master regulator of angiogenesis [89].

4.4. Migration and invasion

Nicotine exposure results in downregulation of the epithelial markers E-cadherin, β-catenin, and the tight-junction protein zonula occludens (ZO-1) with concomitant upregulation of the mesenchymal proteins, fibronectin and vimentin [90,91]. This epithelial-mesenchymal transition (EMT) is a phenomenon associated with increased cell mobility and invasion. Migration and invasion are key events in the process of metastasis. Nicotine treatment either though intraperitoneal injections or through dermal patches also promotes tumor growth and metastasis in immunocompetent mice [91]. Moreover, NNK treatment leads to increased invasion and migration of lung cancer cells via ERK-dependent phosphorylation of calpains [92]. Pharmacological inhibition of ERK or gene silencing of calpains abolishes this response. The pro-metastatic effects of nicotine and its metabolites may contribute to the aggressiveness of SCLC, a lung cancer type highly associated with cigarette smoking [7].

5. nAChR genetic variants in lung cancer

Recent advances in genetic technology have allowed large-scale genome-wide association studies (GWAS) that screen hundreds of thousands of single nucleotide polymorphisms (SNPs) across thousands of subjects [93]. Such studies have implicated variants in the chromosome 15q24–25 region in the development of nicotine dependence and lung cancer. The 15q24–25 locus, spanning 203 kb, contains the genes encoding the α5, α3, and β4 nAChR subunits (CHRNA5/A3/B4) as well as three other genes: the iron-responsive element binding protein 2 (IREB2), an iron regulatory protein; LOC123688, a putative protein of unknown function; and α4 proteasome subunit protein (PSMA4), a peptidase [5].

Genetic association between the CHRNA5/A3/B4 genes and nicotine dependence was first reported in an association study that compared nicotine-dependent smokers versus those who did not present with symptoms of dependence [94]. Multiple SNPs were correlated with nicotine dependence, including rs16969968, located in the CHRNA5 coding region. This polymorphism changes an amino acid from aspartic acid to asparagine at position 398 (D398) in the major cytoplasmic loop of the α5 subunit. This highly conserved aspartate residue is invariant across species, that is, frogs, chickens, rodents, cattle, and nonhuman primates all possess an aspartic acid residue at this location [95]. Follow-up studies showed that one copy of the risk allele confers a 1.3-fold increase in risk for developing nicotine dependence, whereas subjects homozygous for the risk allele have almost a 2-fold increase in risk [96]. These results have now been replicated independently by many groups using a variety of approaches and target populations [95,97–102]. Most of these studies focused on populations of European descent. However, allele frequencies for the chromosome 15 region differ for populations of Asian and African origin. The rs16969968 SNP, in particular, is rare in Asian and African

populations [5]. Nevertheless, chromosome 15q24–25 has been implicated in nicotine dependence and lung cancer in African Americans, providing further support for the role of this genomic locus in smoking and lung cancer across populations [96,103].

Results from analogous large-scale genetic studies in lung cancer converged on the same variants in chromosome 15q24–25 [104–106]. This locus was found to account for 14% of lung cancer cases in a European population [104] and for 18% of cases in an Icelandic population [105]. rs16969968 was again found to be among the SNPs with the strongest disease association [106]. Consistently, a candidate gene study showed that the rs16969968 risk allele is associated with increased risk for lung adenocarcinomas in an Italian population [107]. Two other SNPs highly associated with lung cancer are rs1051730, found in the coding region of CHRNA3, and rs8034191, located in LOC123688 [104,105].

Because the chromosome 15 region is associated with both nicotine dependence and lung cancer, it begs the question of whether lung cancer is directly influenced by the genetic variants or merely the consequence of smoking behaviors. Genetic evidence for the two lines of reasoning exist. Increased lung cancer risk in non-smokers supports the view that the polymorphisms have a direct effect on lung cancer [104]. Additionally, no association was found for other smoking-related cancers such as head and neck cancers including those of the oral cavity, larynx, pharynx, and esophagus [104]. Association with lung cancer also persists even after accounting for cigarette consumption [5]. However, one study did show that cigarette consumption per se might not be a proper measure as individuals who smoke the same number of cigarettes per day exhibit varying levels of carcinogen and toxin exposure [108]. In fact, carriers of the risk variant ingest more tobacco toxins, possibly by inhaling more frequently and more deeply while smoking.

To distinguish between the two possibilities, direct evaluation of the biological function of specific SNPs needed to be performed. A reasonable candidate for initial studies is rs16969968 as it encodes a non-synonymous SNP in CHRNA5. Indeed, heterologous expression of the α5 cDNA containing this SNP, along with α4 and β2, was carried out in HEK293T cells [95]. In this study, agonist-induced changes in intracellular calcium were measured using an aequorin-based luminescence assay. α4β2α5 nAChRs with the asparagine variant exhibited lower maximal response to the nAChR agonist, epibatidine, indicating that the α5 risk allele is associated with reduced function of α4β2α5 nAChRs. This work provides direct evidence that a variant associated with nicotine dependence and lung cancer alters biological function.

Additional mechanistic evidence for the role of nAChR variants in nicotine dependence is provided by work showing that rs3841324, a SNP found in the non-coding region of α5, is associated with altered α5 mRNA levels in the brain [109]. rs3841324 is characterized by an insertion/deletion located upstream of the α5 coding region. In this study, individuals homozygous for the minor allele (deletion) exhibit a 2.9-fold increase in CHRNA5 mRNA levels. rs3841324 is in high linkage disequilibrium with other SNPs in this region. Consistently, these SNPs are also associated with altered CHRNA5 mRNA expression.

Combined analyses of the above biological mechanisms demonstrate that the risk allele of rs16969968 primarily occurs on a low CHRNA5 expression background [109]. Moreover, a combination of low mRNA expression and the presence of the non-risk allele confer protection for both nicotine dependence and lung cancer. In normal lung tissue, rs16969968 also correlated with CHRNA5 mRNA levels (i.e., an inverse relationship was observed between risk allele dosage and mRNA levels) [107]. The same study showed a 30-fold upregulation of CHRNA5 mRNA levels in lung adenocarcinoma compared to normal lung tissue. In contrast, no differences in expression between cancer and normal samples were observed for the other genes in chromosome 15 outside the CHRNA5/A3/B4 gene cluster. Taken together, the aforementioned studies offer two mechanistic bases for the association of nAChR variants with nicotine dependence and lung cancer: altered receptor function and aberrant gene expression.

Consistently, our group showed that the CHRNA5/A3/B4 gene cluster is overexpressed in SCLC cell lines and patient tissues versus normal samples, supporting the hypothesis that these genes play a direct role in this lung cancer type [27]. This overexpression is regulated by the basic helix-loop-helix transcription factor, achaete–scute complex homolog-1 (ASCL1). ASCL1 (also known as ASH1/MASH1) is a member of the Notch signaling pathway and has been implicated in SCLC pathogenesis [110–113]. Further support for a direct role of α3 nAChRs in lung cancer is provided by a study showing that CHRNA3 is a frequent target of aberrant DNA hypermethylation and silencing in lung cancer [114].

6. Perspectives and future directions

More than 1 billion people around the world smoke, resulting in deleterious health consequences. Cigarette smoking and second-hand smoke account for nearly 90% of lung cancer deaths. Aside from the many carcinogens found in cigarettes, nicotine itself promotes lung cancer by activating different nAChR subtypes. The homomeric α7 nAChR as well as the heteromeric α3α5β4 nAChRs appear to be key players in this process.

nAChRs serve as central mediators for various stimuli that promote tumor progression. That multiple stimuli can activate numerous lung cancer signaling pathways may underlie the resistance of this disease to currently available therapies. It may also provide a mechanistic basis for the lower survival rates observed in patients who continue to smoke during chemotherapy [115–117].

The notion that nAChRs function to promote lung carcinogenesis raises questions regarding the safety and appropriateness of nicotine replacement therapies. On the other hand, it also suggests that strategies for molecular therapies targeted against nAChRs may prove to be effective against lung cancer. Delineation of specific nAChR subtypes involved in various types and subtypes of lung cancer could provide highly tailored treatments. Furthermore, the identification of various players in lung cancer signaling provides multiple targets for therapeutic interventions.

Finally, intersecting results from multiple genetic studies point towards a role for the CHRNA5/A3/B4 gene cluster in the development of lung cancer, perhaps through altered gene expression and/or receptor function. The use of cell-based assays and animal models to directly test the effect of the different SNPs on lung cancer development should address the question of whether lung cancer risk is directly influenced by these variants or indirectly influenced by smoking behaviors.

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