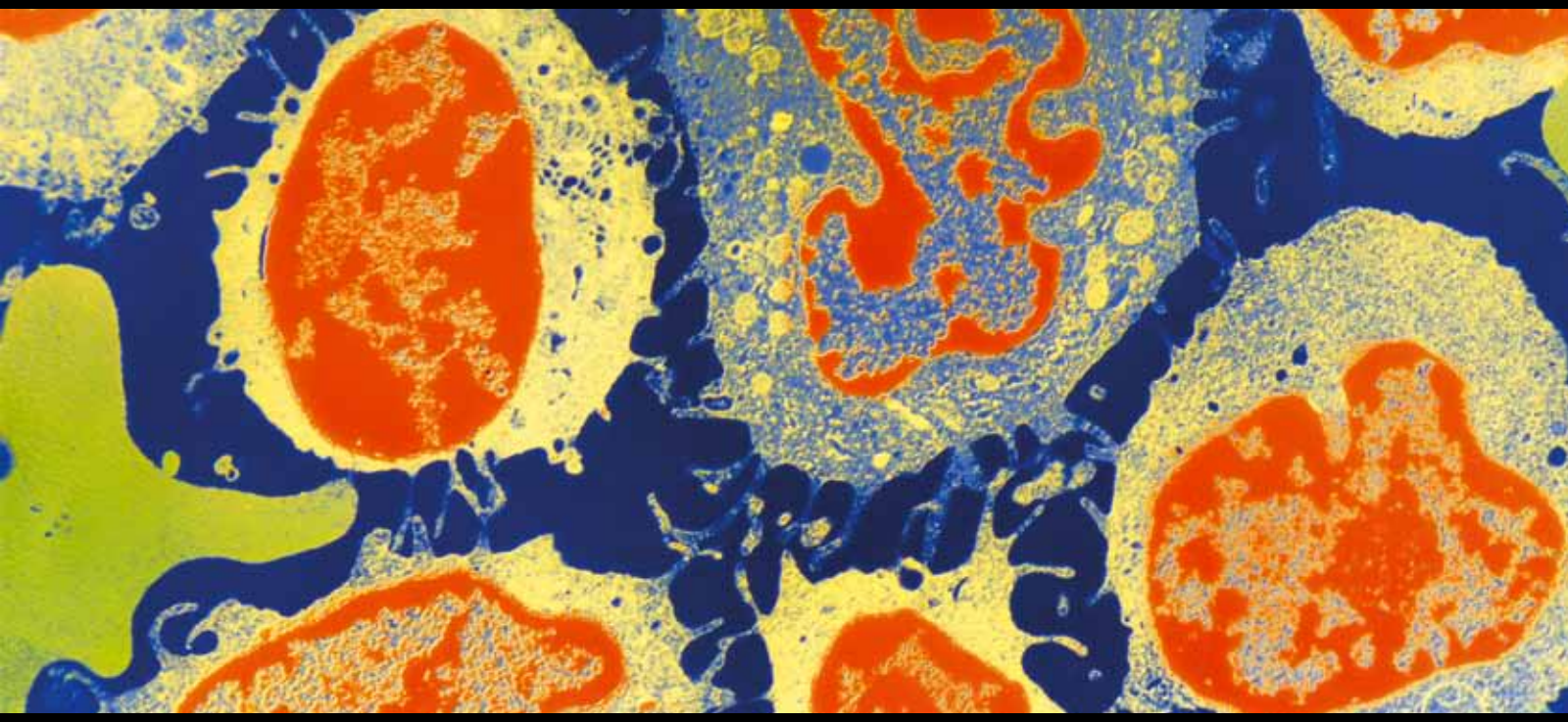
Journal of Oncology

# Cigarette Smoke and Cancer

Guest Editors: Sushant Kachhap, Venkateshwar G. Keshamouni, David Z. Qian, and Aditi Chatterjee



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# Editorial Board

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*Editorial*

# Cigarette Smoke and Cancer

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Increased tobacco smoke exposure positively correlates with a wide variety of cancers including those of lung, breast, pancreas, and prostate cancer. There are about 250 chemicals present in tobacco that have been linked to cancer. Smokeless tobacco, in the form of chewed tobacco leaves, snuff, and betel quid, has also been linked to oral and pancreatic cancers. Recent efforts to decipher mechanisms by which tobacco-derived carcinogens induce various cancers have provided profound insights about signaling pathways that are perturbed by these compounds, leading to oncogenic signaling. This special issue collates reviews and research articles that provide insights about tobacco-induced cancers at molecular, clinical, and epidemiological level.

Emphasizing the use of tobacco as a global health concern, Oppeltz et al. provide a detailed review highlighting the trend towards increased tobacco use and the increasing cancer burden in developing countries. Developing countries, such as Taiwan, may indeed be at risk which is underscored by the study of Lin et al., who evaluated a prospective study cohort and found that habitual cigarette smokers, alcohol consumers, and betel quid chewers have a higher risk of contracting oral cancer. This study finds an alarming 40-fold risk of developing oral cancer in individuals who have all the above habits than controls.

The risk of cancer is not limited to smokers but also affects individuals who are indirectly exposed to tobacco-derived carcinogens. However, the link between paternal smoking and childhood leukemia is not yet clearly established. Using meta-analysis, an interesting review article by Liu et al. draws a link between paternal smoking and childhood acute lymphoblastic leukemia. Pancreatic cancers have been linked to tobacco use. A research article by Lochan et al. further implicates family history as an important factor promoting cancers among smokers. The authors find that individuals with a family history of malignancy are at an increased risk of pancreatic cancer. Furthermore, individuals with a family history of malignancy and who smoke appear to require a lesser degree of tobacco exposure for the development of pancreatic cancer. To curb the use of tobacco, it is necessary to provide professional smoking cessation aid. However, smoking cessation intervention should be tailored depending on the population and ethnicity, and behavioral and cultural differences should be taken into account. A study by Delnevo et al. indicates the heterogeneous nature of tobacco use among South Asian immigrants in the USA. The study drives an important point of segregating tobacco users depending on their country of origin, and not just grouping them as "Asians," for a more reliable understanding into the behavior of tobacco use in this population. A short clinical report by Mazza et al. emphasizes the need for smoking cessation clinics in comprehensive cancer centres to benefit smoker cancer patients.

Understanding tobacco-induced cancers at the molecular level is key for developing biomarkers and therapeutics for early intervention. Chen et al. provide a comprehensive

review about epigenetic and molecular mechanisms that are deregulated by tobacco carcinogens with special emphasis on nicotine, N-nitrosodiethylamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and polyaromatic hydrocarbon. This paper highlights the complex nature of tobacco-induced carcinogenesis and provides recent updates on molecular targets which include receptors, cell cycle regulators, mitogen-activated protein kinases, apoptosis mediators, angiogenic factors, and invasion and metastasis mediators. A research article by Dev et al. explores the molecular mechanism of cigarette-smoke—induced proliferation of lung cells. They found that p-benzoquinone, in aqueous smoke extract, binds and modifies EGFR, preventing its degradation leading to increased EGFR signaling and proliferation. Chaudhry et al., on the other hand, report the effects of brief exposure to tobacco-derived carcinogens, including NNK, on cellular activity, morphology, and gene expression of bronchial epithelial cells. Knowledge gained from *in vitro* work has been extended to *in vivo* models. Recent advances in transgenic and knockout animal models have provided unprecedented opportunity to selectively perturb molecular pathways and understand its role in tobacco-induced carcinogenesis. Zheng et al. provide a comprehensive review of our current understanding of pathways altered by NNKs using such animal models.

Search for reliable biomarkers for early detection of lung and oral cancer is an active area of research. Recent advances in tools to probe epigenetic changes in the DNA have included DNA methylation in the repertoire of biomarkers of predictive and prognostic importance. Using MethyLight assays, Salskov et al. investigated hypermethylation in lung tissues in a cohort of smokers and nonsmokers for nineteen gene promoters. Their data suggests hypermethylation of CCND2 could reflect smoking-induced precancerous changes in the lung. Although several compounds in tobacco are proven carcinogens, nicotine, the main addictive compound in tobacco, is not carcinogenic. Two review articles, one by Singh et al. and the other by Lee et al., describe the role of nicotine in carcinogenesis. While Singh et al. examine the historical data connecting nicotine tumor progression with updates on recent efforts to target the nicotinic acetylcholine receptors to combat cancer, Lee et al. provide recent updates on the assembly, activity, and biological functions of nicotinic receptors, with current understanding regarding developments in the therapeutic application of nicotinic receptor ligands. Carcinogens present in cigarette smoke are not always intrinsic to tobacco leaves; fertilizer-derived carcinogens and microbial toxins could also contribute to carcinogenesis. Review articles by Zaga et al. about radioactive carcinogens, Pb-210 and Po-210, which accumulate in tobacco leaves, and by Pauly et al. about microbes and microbial toxins in cigarettes, provide important insight into the interesting cancer-promoting milieu of tobacco smoke.

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*Review Article*

# Nicotinic Acetylcholine Receptor Signaling in Tumor Growth and Metastasis

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Cigarette smoking is highly correlated with the onset of a variety of human cancers, and continued smoking is known to abrogate the beneficial effects of cancer therapy. While tobacco smoke contains hundreds of molecules that are known carcinogens, nicotine, the main addictive component of tobacco smoke, is not carcinogenic. At the same time, nicotine has been shown to promote cell proliferation, angiogenesis, and epithelial-mesenchymal transition, leading to enhanced tumor growth and metastasis. These effects of nicotine are mediated through the nicotinic acetylcholine receptors that are expressed on a variety of neuronal and nonneuronal cells. Specific signal transduction cascades that emanate from different nAChR subunits or subunit combinations facilitate the proliferative and prosurvival functions of nicotine. Nicotinic acetylcholine receptors appear to stimulate many downstream signaling cascades induced by growth factors and mitogens. It has been suggested that antagonists of nAChR signaling might have antitumor effects and might open new avenues for combating tobacco-related cancer. This paper examines the historical data connecting nicotine tumor progression and the recent efforts to target the nicotinic acetylcholine receptors to combat cancer.

## 1. Introduction

Smoking is a major risk factor associated with the development and progression of a variety of cancers [1]. Smoking is estimated to account for approximately 4-5 million deaths worldwide and approximately 443,000 deaths each year in the United States alone [2, 3]. Sufficient evidence has accumulated to conclude that tobacco smoking caused cancers not only of the lung, but also of the lower urinary tract including the renal pelvis and bladder, upper aero-digestive tract including oral cavity, pharynx, larynx, and esophagus, and pancreas [2, 4]. Recent lines of evidence have showed that smoking tobacco can also cause cancers of the nasal cavity, paranasal sinus, nasopharynx, stomach, liver, kidney, cervix, uterus, breast, adenocarcinoma of the esophagus, and myeloid leukemia [2]. Of the thousands of chemicals in tobacco smoke, polycyclic aromatic hydrocarbons and nicotine-derived nitrosamines have been identified as the major and potent carcinogens [5, 6]. The metabolites of these agents form DNA adducts and cause mutations in vital genes like Rb, p53, and K-Ras in smokers [7–9].

While the induction of these cancers is mediated by tobacco-specific nitrosamines as well as other carcinogens present in the tobacco smoke, it is becoming clear that signaling through the nicotinic acetylcholine receptors contribute to the growth, progression, and metastasis of a variety of cancers. Nicotine, which is the major addictive component of tobacco smoke, acts through nicotinic acetylcholine receptors (nAChR) [9–11], but is not thought to be carcinogenic. The expression of nAChRs in central and peripheral nervous system is associated with smoking dependence and addiction [12]. It was generally believed that nAChRs are only expressed in nervous system and at neuromuscular junctions (muscle type nAChRs). However, the discovery of widespread expression of nAChRs in mammalian cells, including cancers, suggested its direct role in cancer progression [13–15]. This paper deals with certain aspects of nicotinic receptor signaling in nonneuronal

cells that lead to increased cell proliferation and survival, angiogenesis, tumor growth, and metastasis.

## 2. Nicotinic Acetylcholine Receptor Expression in Nonneuronal Cells

nAChRs are a complex of five subunits forming hetero- or homopentamers to form a central ion channel [16, 17]. The neuronal nAChRs can be homomeric composed of $\alpha 7$, $\alpha 8$, or $\alpha 9$ subunits or with the combinations of $\alpha 2$–$\alpha 6$ or $\alpha 10$ subunits with $\beta 2$–$\beta 4$ subunits (heteromeric nAChRs). The muscle type nAChRs may be comprised of combinations of $\alpha 1$ subunits with $\beta 1$, $\gamma$, $\delta$, or $\varepsilon$ subunits [18]. Both neuronal as well as muscle nAChR families are found to be expressed in cancer cells [19]. Nicotine mimics acetylcholine by binding as an agonist to $\alpha$ subunit of nAChRs [10]. Nicotine binds with higher affinity to heteromeric $\alpha 4\beta 2$-nAChRs than to $\alpha 7$-nAChRs [20]. Higher binding to $\alpha 4\beta 2$-nAChRs results in desensitization of the receptor, which could be the reason that $\alpha 7$-nAChR is the major stimulator of cancer development and progression *in vivo*. In addition to nicotine, tobacco-specific nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) can also bind to $\alpha 7$-nAchR, and *N*-nitrosonornicotine (NNN) binds to heteromeric $\alpha\beta$-nAChRs [21]. The affinity of NNK for the $\alpha 7$-nAChR was found to be 1,300 times higher than nicotine, whereas the affinity of NNN for heteromeric $\alpha\beta$-nAChRs was 5,000 times higher than that of nicotine [21, 22].

Since the discovery of ubiquitous presence of nAChRs in mammalian cells, studies from many laboratories have linked nAChRs with various pathological conditions including tumor growth and angiogenesis [13, 23]. In earlier studies, nicotine was found to stimulate endothelial-cell proliferation via nAChR at concentrations lower than those obtained in blood after smoking [24]. As described in the later part of this paper, many studies have correlated the exposure of nicotine or other tobacco smoke components with induction of pathological neovascularization through the activation of nAChR [23, 25]. Studies from our laboratory have suggested that nicotine can enhance the growth and metastasis of pre-established lung tumors [26]. Altogether, these studies proposed the involvement of tobacco smoke components in various aspects of tumorigenesis and vascular dysfunctions in smokers. Extensive research by many groups has successfully associated the physiological effect of nicotine and its derivatives with the direct activation of nAChRs. Small cell lung carcinoma (SCLC) pulmonary neuroendocrine cells (PNECs) and SCLC cells express high levels of the $\alpha 7$-nAChR, whereas heteromeric nAChRs were undetectable [27, 28]. At the same time, both hetero- and homomeric nAChRs are found to be expressed in nonsmall cell lung carcinoma cells of different histologic subtypes [19, 29]. Recently, differential expression pattern of *ACHR* subunit gene was studied in NSCLC patients who were smokers or never smokers. Higher expression of *CHRNA6* and *CHRNB3* combination was correlated with NSCLCs in nonsmokers, whereas lower expression was correlated with NSCLCs in smokers. Additionally, increased expression of *CHRNA1, CHRNA5, and CHRNA7* subunit genes was correlated with short-term exposure to nicotine [30]. Nicotine stimulation contributed towards the growth of human mesothelioma cells. Human biopsies of mesothelioma as well as of normal pleural mesothelial cells were found to express functional $\alpha 7$-nAChR [31, 32]. Studies from the Russo laboratory have shown that inhibition of nAChRs by $\alpha$-cobratoxin ($\alpha$-CBT) can inhibit the growth of A549 tumors in immunocompromised mice [33]. These findings strengthen the hypothesis that modulation of nAChRs upon chronic exposure to tobacco may contribute to the development and progression of cancer. In the following sections, we will summarize the findings to support the hypothesis.

## 3. nAChRs Signaling in Tumor Growth and Survival

Attempts have been made to elucidate the molecular events that mediate nicotine-induced cell proliferation. Activation of nAChR through nicotine or NNK has been found to activate protein kinase C (PKC), the serine/threonine kinase Raf-1, the mitogen-activated kinases ERK1 and ERK2, and the transcription factors FOS, JUN, and MYC through the selective activation of $\alpha 7$-nAChR in SCLC [34]. Studies also demonstrated the stereospecificity of nAChRs towards (−)-nicotine. It has been reported that (−)-nicotine stimulated tumor cell proliferation via secretion of the neurotransmitter serotonin, and the growth stimulatory effect of nicotine or NNK could be blocked by selective serotonergic receptor antagonists [27, 35, 36]. In a recent report, the effects of acute and repetitive exposure to nicotine was shown to induce a neuronal-like appearance in N417 SCLC cell line, which produced bigger and more vascularized tumors in mice through activation of CXCR4/CXCL12 axis. A prominent increase in the expression of CXCR4 was observed in nAChR-dependent manner in nicotine-treated cells [37]. NSCLC cell lines from large-cell carcinoma, squamous-cell carcinoma, and adenocarcinoma, all showed the activation of PI3K-AKT pathway and NF-$\kappa$B activation in response to nicotine or NNK treatment [38, 39]. In addition, frequent loss of the tumor suppressor gene FOXO3a was reported in carcinogen-induced lung adenocarcinoma. In NNK-treated lung cancer cells, restoration of FOXO3a in FOXO3a-deficient cells increases sensitivity to apoptosis caused by a DNA-damaging intermediate of NNK. This study proposed that FOXO3a might play a role in lung adenocarcinoma suppression by providing a protective response to carcinogenic stress [40].

Experiments from our laboratory have shown that nicotine stimulation affects various components of cell cycle regulatory machinery [26, 29, 41]. Exposure to nicotine resulted in activation of Raf-1, induction of cyclin D and cyclin E-associated kinase activity as well as Rb phosphorylation, which led to the dissociation of E2F1 from Rb. Further, it was observed that stimulation with nicotine caused the dissociation of Rb from E2F-responsive proliferative promoters (*cdc6 and cdc25A*), while there were

increased amounts of E2F1 bound to them. These molecular events were correlated with increased proliferative effects of nicotine in NSCLC cell lines A549 (human bronchioalveolar carcinoma), NCI-H23, NCI-H441 (lung adenocarcinoma), and NCI-H226 (pleural effusion squamous cell carcinoma) as well as on primary normal human bronchial epithelial cells (NHBEs), small airway epithelial cells (SAECs), human aortic endothelial cells (HAECs), and human microvascular endothelial cells from lung (HMEC-Ls). The mitogenic effects of nicotine were abrogated by $\alpha 7$ subunit antagonists, $\alpha$-bungarotoxin, and methylallyl aconitine (MAA), whereas it was unaffected by $\alpha$-lobeline ($\alpha 4\beta 2$ subunit inhibitor) or dihydro $\beta$-erythoidine (DH$\beta$E; $\alpha 3\beta 2$ and $\alpha 4\beta 2$ subunit inhibitor), suggesting that $\alpha 7$ subunits primarily mediated the mitogenic effects of nicotine in NSCLC cells. We have further illustrated that upon nicotine stimulation, the scaffolding protein $\beta$-arrestin-1 forms a complex with nonreceptor tyrosine kinase-Src and gets recruited to the nAChRs. Depletion of $\beta$-arrestin-1 or Src prevented nicotine-induced cell proliferation. These results suggested that $\alpha 7$-nAChR-mediated stimulation of cell proliferation is through a $\beta$-Arrestin-1-Src signaling axis in NSCLC [41]; (see also Figure 1).

Other than lung cancer, activation of $\alpha 7$-nAChR and heteromeric nAChRs expressing $\alpha 3$ and $\alpha 5$ subunits have been reported in oral and esophageal keratinocytes [22]. Similar to lung cancer cells, NNK was found to bind with high affinity to $\alpha 7$-nAChR, whereas NNN was found to bind to heteromeric nAChRs with higher affinity [22]. Esophageal cancer-Het-1A cells stimulated with NNK or NNN showed increased mRNA transcripts and expression of PCNA and Bcl-2, and transcription factors GATA3, NF-$\kappa$B, and STAT1. However, induction of Ras-Raf-ERK1-ERK2 cascade, the JAK2-STAT3 pathway and NF-$\kappa$B activation was associated with enhanced cell proliferation through these nitrosamines in immortalized oral epithelial cells [22]. In addition, chronic exposure of nicotine or environmental tobacco smoke on oral keratinocytes selectively upregulated $\alpha 5$- and $\alpha 7$-nAChR subunits, resulting in intensified signaling responses to nicotine [42].

The secreted mammalian Ly-6/urokinase plasminogen activator receptor-related protein-1 (SLURP-1) is recently identified as an endogenous ligand for the $\alpha 7$ subunit of the nicotinic acetylcholine receptor (nAChR). The expression levels of SLURP1 and SLURP2 (secreted mammalian Ly-6/urokinase plasminogen activator receptor-related protein-2) were reduced in NNK-treated cells. Transfection of the cells with SLURP1 or SLURP2 cDNA reduced the nitrosamine-induced colony formation in soft agar while inhibiting the growth of NNK-transformed keratinocytes in mouse xenografts. SLURP1 bound to $\alpha 7$-nAChR and SLURP2 bound to nAChRs expressing the $\alpha 3$ subunit [22, 43]. Similar results were demonstrated recently where HT-29 human colon cancer cells treated with nicotine resulted in increased cell proliferation and a marked reduction in the protein expression of SLURP1 via $\alpha 7$-nAChRs activation [44]. Recently, nicotine mediated upregulation of FOXM1 expression was found in primary oral keratinocytes which was associated with induction of genomic instability. A centrosomal protein CEP55 as well as a DNA helicase and putative stem cell marker HELLS, were found to be novel targets of nicotine-induced FOXM1 expression and correlated with oral cancer progression [45].

A role of nAChR has been demonstrated in breast cancer progression as well. Experiments with human mammary epithelial-like MCF10A or cancerous MCF7 cells revealed that treatment of these cells with nicotine enhances the activity of protein kinase C (PKC) alpha with cdc42 as a downstream target for nicotine-induced proliferation and migration [46]. It has also been suggested that nicotine-induced proliferation of human breast cancer cell is dependent on $\alpha 9$-nAChR and cyclin D3 expression [47]. The effects of nicotine on a population of cancer stem cells in MCF-7 human breast cancer cells were examined, using aldehyde dehydrogenase (ALDH) as a stem cell marker. This study found that nicotine increases the stem cell population via $\alpha 7$-nAChR and the PKC-Notch dependent pathway [48].

Apart from direct responses through nAChRs, indirect signaling events may also contribute to nicotine-induced tumor growth and survival. Since nAChRs are cation channels, it can stimulate signaling cascades by the influx of Ca2+ through the opened $\alpha 7$-nAChR [49]. Ca2+ channel blockers are shown to significantly reduce DNA synthesis in response to nicotine or NNK in SCLCs [49]. Also, nAChR-mediated systemic increase in stress neurotransmitters, adrenaline, and noradrenaline, which are $\beta$-adrenergic agonists, are also shown to stimulate $\beta$-adrenergic receptor-initiated cAMP signaling and transactivation of EGFR cascade through EGF secretion in NNK-treated small airway epithelial cells [50, 51]. Nicotine is found to induce systemic or cellular increase in noradrenaline and significantly enhance the growth and angiogenesis of pancreatic, gastric, and colon cancer-xenografts with increased expression of ERK1-ERK2, COX2, prostaglandin E2, VEGF, and transactivation of $\beta$-adrenergic as well as EGFR signaling in colon cancer cells [52–55]. Activation of ERK1-ERK2 and STAT3 in response to nicotine has also been reported in bladder cancer cells downstream of nAChRs and $\beta$-adrenergic receptors [56]. Importantly, apart from nAChRs, direct interaction of NNK with $\beta$-adrenergic receptor has been proposed as a novel mechanism, which may significantly enhance the high cancer-causing potential of these nitrosamines [50, 57]. Similar to the activation via neurotransmitters, NNK binding to $\beta$-adrenergic receptor was also found to activate adenylyl cyclase-cAMP-PKA-CREB cascade and transactivation of EFGR [58]. Additionally, an additive effect of estrogen receptors and nAChRs was also demonstrated in promoting the growth of A549 tumors in athymic nude mice. Cotreatment of nicotine and estradiol resulted in increased cell proliferation as well as VEGF secretion from cancer cells, leading to increased tumor growth as well as microvascular density within the tumor [59]. Recently, the chronic exposure to estrogen and NNK was shown to have synergistic effects on cell proliferation and production of noradrenaline and adrenaline, by upregulating $\alpha 7$-nAChRs in immortalized small airway epithelial cells [60].

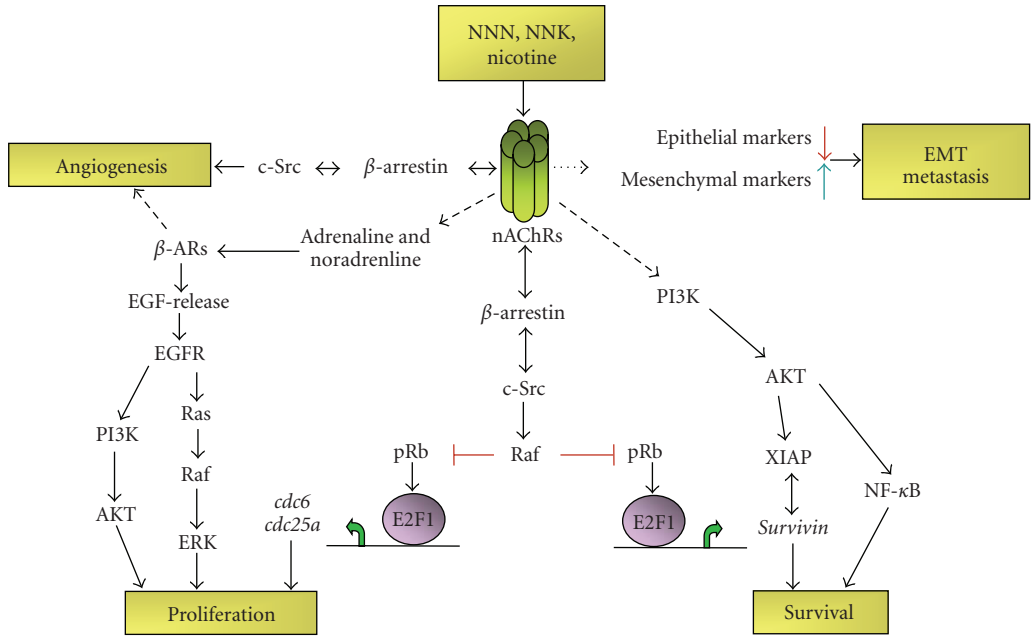


Figure 1: A schematic of nAChR-mediated regulation of diverse tumorigenic processes. nAChRs are activated by tobacco smoke components like NNN, NNK, and nicotine with different affinity. Induced nAChRs activate several downstream signaling pathways involved in cell proliferation, inhibition of apoptosis, metastasis, and angiogenesis in a variety of cancer and primary cells. Agonist binding to nAChR forms complex with $\beta$-arrestin and Src and results in Raf-1 activation. Activated Raf-1 phosphorylates and inactivates Rb tumor-suppressor-function. These in turn results in E2F-1-mediated transcriptional upregulation of target genes involved in cell proliferation, angiogenesis, and inhibition of apoptosis. Downstream effect of nAChR activation is also indirectly supported by the activation of $\beta$-adrenergic receptor ($\beta$-AR) signaling. Nicotine exposure directly results in metastatic dissemination of primary tumor by inducing epithelial to mesenchymal transition (EMT) in cancer cells.

## 4. nAChRs Signaling in Cell Survival and Resistance to Apoptosis

In addition to the effect on tumor growth, epidemiological and clinical data implicate that in patients with cancer, continued smoking causes resistance to therapy by blocking the induction of apoptosis. Various studies have linked the activation of nAChR resulting in inhibition of apoptotic pathways. In SCLC cells, NNK was shown to phosphorylate Bcl-2 at Ser70 which promoted its interaction with c-Myc that significantly enhanced the half-life of the c-Myc protein [61]. This functional cooperation of Bcl2 and c-Myc resulted in promoting cell survival and proliferation. This effect could be blocked by the PKC inhibitor staurosporin, the ERK1-ERK2 inhibitor PD98059 or silencing of MYC [61, 62]. Additionally, mesothelioma cells also showed nicotine-stimulated proliferation through $\alpha$7-nAChR-mediated $Ca^{2+}$-dependent activation of the ERK1-ERK2 cascade and inhibited apoptosis by induction of NF-$\kappa$B and phosphorylation of BAD at Ser112 (Bcl-2 antagonist of cell death) [32]. In NSCLCs, constitutive activation of AKT is associated with lung cancer cell survival and resistance to chemotherapy and radiation [63]. Similarly, nicotine or NNK exposure displayed AKT-mediated growth and NF-$\kappa$B-mediated resistance to apoptosis in human airway epithelial cells as well as lung cancer cells [38, 39]. Further, activated AKT could directly phosphorylate Bax *in vitro* in nicotine treated cells. Treatment of cells with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 or specific depletion of AKT was shown to block both nicotine-induced Bax phosphorylation and cisplatin resistance in NSCLC cells [64].

In addition to these signaling events, results from our laboratory revealed a significant role for the IAP proteins XIAP and survivin in nicotine-mediated chemoresistance in NSCLCs *in vitro*. Chromatin immunoprecipitation assays demonstrated that nicotine stimulation caused an increased recruitment of E2F1 and concomitant dissociation of retinoblastoma tumor suppressor protein (Rb) from survivin promoter in NSCLC cells [65]. Moreover, ablation of E2F1 levels caused abrogation of survivin expression and protective effects of nicotine against cisplatin-induced apoptosis in A549 cells. In the above study, chemoprotective effect of nicotine was found to be mediated through $\alpha$3/$\beta$4-nAChR activation and could be abrogated by agonists of these subunits. It was also found that nicotine stimulation enhanced the levels of XIAP at the protein level. Nicotine induces the activation of Akt, which is known to phosphorylate XIAP and prevent its proteasome-mediated degradation [66]. In agreement with this, an Akt inhibitor could abrogate the antiapoptotic effects of nicotine in A549 cells [65].

In other studies, the cooperative effect of nicotine and NNK was investigated for their transforming ability in various lung epithelial or cancer cells. Exposure to nicotine or the combination of nicotine and NNK for one week augmented

Bcl-2 expression, accompanied by an increased resistance to cisplatin-induced apoptosis [67]. This study also showed that the combination treatment promoted cell proliferation and anchorage-independent growth as compared to NNK exposure alone [67]. In another study, nicotine was demonstrated to mediate prosurvival activity by Mcl-1 phosphorylation. Nicotine-induced Mcl-1 phosphorylation significantly enhanced the half-life of Mcl-1, which conferred long-term survival potential [68]. Specific depletion of Mcl-1 by RNA interference blocked nicotine-stimulated survival and enhanced apoptotic cell death [67]. Nicotine-mediated activation of $\alpha$7-nAChR has also been linked with the expression of PPAR$\beta/\delta$ protein by inhibiting AP-2$\alpha$ protein expression and DNA binding activity to the PPAR$\beta/\delta$ gene promoter [69]. Sp1 was found to modulate this process. $\alpha$7-nAChR antagonist and short interfering RNA against $\alpha$7-nAChR as well as inhibitors of phosphatidylinositol 3-kinase (PI3K; wortmannin and LY294002) and mammalian target of rapamycin (mTOR; rapamycin) blocked the expression of PPAR$\beta/\delta$ protein demonstrating a novel mechanism by which nicotine could promote human lung carcinoma cell growth [69]. These studies show that signaling through the nAChRs could promote cell proliferation and survival, utilizing multiple signaling cascades.

## 5. nAChRs and Tumor Angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a complex multistep process involved in a number of physiological processes such as wound healing, embryogenesis and reproduction. In addition, angiogenesis is necessary for the sustained growth of the primary tumor as well as metastatic dissemination. Nicotine has been shown to enhance angiogenesis in many experimental systems and animal models. The proangiogenic activity of nicotine is mediated by nicotinic acetylcholine receptors, particularly $\alpha$7 subunit. The pioneering study by Villablanca (1998) demonstrated the ability of nicotine to induce endothelial cell proliferation [24]. This observation was followed by the elegant studies from the John Cooke's laboratory suggesting a cholinergic pathway for nicotine-induced angiogenesis where they demonstrated complete inhibition of endothelial network formation using nonselective nAChR antagonist mecamylamine in an *in vitro* angiogenesis model [25]. Although several nAChR isoforms are expressed on endothelial cells, a similar inhibition was obtained only with the selective $\alpha$7-nAChR antagonist $\alpha$-bungarotoxin, confirming the specific involvement of $\alpha$7-nAChR. Further, *in vivo* pharmacological inhibition of nAChR and a genetic disruption of $\alpha$7-nAChR expression significantly inhibited inflammatory angiogenesis and reduced ischemia-induced angiogenesis and tumor growth. They also provided anatomic and functional evidence for nicotine-induced angiogenesis and arteriogenesis when they observed that nicotine accelerated the growth of tumor and atheroma in association with increased neovascularization [23].

Nicotine increased endothelial-cell growth and tube formation *in vitro,* and accelerated fibrovascular growth *in vivo*. In a mouse model of hind-limb ischemia, nicotine increased capillary and collateral growth, and enhanced tissue perfusion. These effects of nicotine were mediated through nicotinic acetylcholine receptors at nicotine concentrations that are pathophysiologically relevant and suggested a possible role for the endothelial production of nitric oxide, prostacyclin, and vascular endothelial growth factor [70–74]. Nicotine has been demonstrated to stimulate postnatal angiogenesis, having an antiapoptotic effect on endothelial cells. It was observed that nicotine stimulated postnatal vasculogenesis on endothelial progenitor cells (EPCs) [75]. The effect of nicotine on EPC survival was significantly enhanced under serum starvation. Furthermore, the antiapoptotic effect of nicotine was blocked completely by nicotinic acetylcholine receptor (nAChR) antagonist hexamethonium bromide [75].

Recent studies have shown that apart from cigarette smoking, exposure to secondhand smoke also could induce angiogenesis. A positive correlation between secondhand smoke exposure and concentrations of nicotine in the body was established after analyzing twenty-two studies measuring the biological effects of nicotine [76]. Further, it was found that the levels of nicotine exposure from secondhand smoke were comparable to those of active smokers. In a mouse model where Lewis lung cancer cells were implanted subcutaneously into mice, which were then exposed to sidestream smoke (SHS) or clean room air and administered vehicle or mecamylamine (an inhibitor of nAChR); SHS significantly increased tumor size, weight, capillary density, VEGF, and MCP-1 levels, and circulating endothelial progenitor cells (EPC). Mecamylamine partially inhibited the effects of SHS on these angiogenic processes and nearly abolished the effects of SHS on tumor capillary density suggesting that nicotine mediated the effects of SHS on tumor angiogenesis and growth [77].

Several recent studies have implicated that nicotine-induced angiogenesis could be mediated by growth stabilization and transmigration of endothelial progenitor cells (EPC) [75, 78, 79]. Nicotine accelerated the growth of syngenic colon cancer CMT93 cells when grown subcutaneously in mice by inducing angiogenesis via bone marrow derived EPCs [78]. To determine if the angiogenic effects of nicotine is mediated by EPC mobilization, Heeschen et al. used a model of mouse parabiosis and found that nicotine enhances EPC mobilization into the vasculature of the ischemic tissue. This effect may be due to the direct actions of nicotine on EPC proliferation, migration and/or mobilization as suggested by *in vitro* models [80] and plasma markers used in the investigation [79]. They also noticed that in the absence of acute ischemia, nicotine did not stimulate EPC mobilization. The activation of nAChRs in response to ischemia induced the release of proangiogenic factors like VEGF and stem cell derived factor-1, both of which are regulated by hypoxia, which in turn facilitates EPC mobilization [81]. Evidence from another study also demonstrated that nicotine promotes angiogenesis via stimulation of nAChR-dependent endothelial cell migration. nAChR antagonism not only abolished nicotine-induced human microvascular endothelial cells (HMVEC) migration but also abolished

migration induced by bFGF and attenuated migration induced by VEGF. Transcriptional profiling identified gene expression programs which were concordantly regulated by all 3 angiogens (nicotine, VEGF, and bFGF), a notable feature of which includes corepression of thioredoxin-interacting protein (TXNIP), endogenous inhibitor of the redox regulator thioredoxin. Furthermore, TXNIP repression by all 3 angiogens induced thioredoxin activity. Interestingly, nAChR antagonism abrogates growth factor (VEGF- and bFGF-) mediated induction of thioredoxin activity suggesting the requirement of nAChR activation in endothelial cell migration, a key angiogenesis event [82].

The proangiogenic effects of nicotine have been found to be mediated by $\alpha$7-nAChR on endothelial cells by activating ERK/MAP kinase pathway, PI3 kinase/Akt pathway, and NF-$\kappa$B [23, 25, 83, 84]. Further, nicotine has been shown to induce the proangiogenic factors like VEGF and HIF-1$\alpha$ in NSCLC cell lines [85]. Pharmacologically blocking nAChR-mediated signaling cascades, including the Ca2+/calmodulin, Src, protein kinase C, PI3K/Akt, MAPK/ERK1/2, mTOR pathways, significantly attenuated nicotine-induced upregulation of HIF-1$\alpha$. These proangiogenic and invasive effects of nicotine were partially abrogated by depleting HIF-1$\alpha$ using siRNA techniques. Additionally, nicotine could promote angiogenesis of gastric cancers by upregulating COX2 and VEGFR2 [86]. Nicotine also enhanced the activity of matrix metalloproteinase 2 and 9 and expression of plasminogen activators in a COX2 and VEGFR2-dependent manner. The proangiogenic effect of nicotine has been shown to be dependent on Src activity by our laboratory [41]. The inhibition of Src, using chemical inhibitors or siRNA has been shown to inhibit endothelial cell proliferation, migration, and angiogenic tubule formation on matrigel. As mentioned earlier, studies from our laboratory suggest that the scaffolding protein $\beta$-arrestin-1 causes the activation of Src. Oligomeric complex comprising of nAChR, $\beta$-arrestin-1, and Src is vital for nAChR signaling. In addition, depletion of $\beta$-arrestin-1 caused abrogation of endothelial cell proliferation and angiogenic tubule formation [29, 41]. These data suggest that nicotine behaves in a manner analogous to growth factors and induces cell cycle progression in endothelial cells.

## 6. nAChRs in EMT and Tumor Metastasis

Epithelial to mesenchymal transition (EMT) is a biological process that allows a polarized epithelial cell, which normally interacts with the basement membrane through its basal surface, to undergo multiple biochemical changes with a signature of more advanced and less differentiated cancer that allow it to assume a mesenchymal phenotype. This enhanced migratory capacity, invasiveness, resistance to apoptosis, and greatly increased production of ECM components [87–89]. This process results in degradation of basement membrane and the formation of a mesenchymal like cell, which can migrate away from the epithelial layer in which it originated [88]. Epithelial to mesenchymal transition (EMT) is involved in tumor progression from noninvasive tumor cells into metastatic carcinomas. Recent studies from our laboratory demonstrated that nicotine can induce invasion and migration in cell lines derived from lung cancer, breast cancer, and pancreatic cancer via $\alpha$7-nAChR-mediated signal transduction pathways [90]. The proinvasive effects of nicotine were mediated by $\alpha$7-nAChR in lung cancer cells while $\alpha$7-nAChR and Dh$\beta$E sensitive nAChRs mediated invasion of breast cancer cells. Nicotine was also found to inhibit anoikis in lung airway epithelial cells. Further, nicotine could induce changes in gene expression consistent with EMT. Long-term treatment of lung cancer and breast cancer cells with nicotine was found to diminish levels of epithelial markers namely $\beta$-catenin and E-cadherin and upregulate mesenchymal proteins like fibronectin and vimentin, indicative of disruption of cell-cell contacts and increased motility [90].

In addition to facilitating EMT, nicotine and NNK have been shown to affect various aspects of tumor cell invasion and migration. For example, both nicotine and NNK are shown to promote the invasion of NSCLC by phosphorylation of $\mu$ and m-calpains [62]. Several lines of evidence show that calpain-mediated proteolysis mediates various aspects of cell physiology including cell migration and invasion. Nicotine was found to induce phosphorylation of both $\mu$ and m-calpains via $\alpha$7-nAChR; the binding of nicotine to $\alpha$7-nAChR in turn was found to activate Src and PKC-iota, leading to enhanced invasion and migration of NSCLC cell line H1299. Similarly, NNK also could promote invasion and migration through phosphorylation of $\mu$ and m-calpains in a $\alpha$7-nAChR-dependent fashion [62].

Several observations in patients suggest that those exposed to tobacco carcinogens are more likely to develop larger, more vascularized tumors with a high propensity for metastatic spread and resistance to chemotherapy [90]. About 30% of lung cancer patients who are smokers continue to smoke after they have been diagnosed [91], which might result in increased adverse medical consequences such as increased tumor progression, development of a second cancer, greater recurrence, greater cancer-related mortality and reduced quality of life [92, 93]. While these studies demonstrate a role for tobacco carcinogens in the initiation, growth, and progression of cancers, the relative contribution of nicotine by itself to these processes is not well explored. A recent study from our laboratory demonstrated that nicotine by itself can induce the growth and metastasis of tumors in immunocompetent mice, independent of other tobacco carcinogens [26]. Nicotine administered either intraperitonially or by commercially available transdermal patches could substantially promote tumor growth. Similar effects were observed on implanted tumors as well as tumors induced by tobacco carcinogen, NNK. Furthermore, mice exposed to nicotine showed significantly enhanced lung metastasis as well as tumor recurrence after surgical removal of the primary tumor, indicating that nicotine can enhance the growth and metastasis of pre-established lung tumors [26]. As mentioned earlier, repetitive exposure to nicotine on SCLC-N417 cells resulted in neuronal-like appearance along with increased adhesion to the extracellular matrix. These changes were accompanied by enhanced migration through

collagen matrices and adhesion to and transmigration across lymphatic endothelial cell monolayers [37].

Accumulating evidence from epidemiological studies suggest a strong association between smoking and pulmonary metastatic disease in women with breast cancer [94]. In a murine model of metastatic mammary cell cancer, cigarette smoke exposure was associated with an increase in the total pulmonary metastatic burden providing experimental support for an adverse effect of smoking on the metastatic process and suggesting a possible mechanism for smokers' increased breast cancer mortality [95]. In addition, it was observed that cigarette smoking was correlated with increased lymph node metastases at mastectomy in women older than 50 years of age suggesting that tobacco usage may potentiate the early spread of malignant disease [96]. Although numerous studies have indicated the role of nicotine exposure in tumor promotion, little is known about the molecular mechanisms by which nicotine promoted breast tumor development, especially on the metastatic process of breast cancer. At least four different subunits of nAChRs including $\alpha$5, $\alpha$7, $\alpha$9, and $\beta$4 are shown to be expressed in breast cancer cells [46]. It has been demonstrated that in addition to proliferative effect, nicotine promoted migration of breast cell lines (mammary epithelial cell line MCF10A and breast cancer cell line MCF7) through a signaling cascade involving PKC activation and its downstream effector cdc42 [46]. Exposure to nicotine has shown to increase the expression of $\alpha$9-nAChR in breast cancer cells [47, 97]. Studies using a soft agar transforming assay and a mouse xenograft model demonstrated that noncancerous human breast epithelial cell line, MCF10A, could be neoplastically transformed by exposure to either a cigarette smoke condensate or the tobacco specific carcinogen, NNK [98, 99]. In a recent study, $\alpha$9-nAChR expression was silenced in MDA-MB-231 breast cancer cells which resulted in reduced proliferation and tumorigenic potential in both *in vitro* and *in vivo* assays, indicating the role of $\alpha$9-nAChR in breast carcinogenesis [100].

Cigarette smoking has recently been recognized as a risk factor for gastric cancer [101] and long-term exposure of nicotine-induced EMT like changes in gastric cancer cell lines by activating Erk/5-Lox signaling pathway [102]. A study on the association between cigarette smoking and pancreatic cancer showed that smokers had a significantly higher risk (70%) of developing pancreatic cancer compared to nonsmokers [103–105]. Accumulating evidence suggests that nicotine induces expression of osteopontin, a secreted phosphoprotein that confers on cancer cells a migratory phenotype and activates signaling pathways that induce cell survival, proliferation, invasion, and metastasis. Rats exposed to cigarette smoke showed a dose-dependent increase in pancreatic osteopontin expression. In addition, analysis of cancer tissues from invasive pancreatic ductal adenocarcinoma (PDA) patients, the majority of whom were smokers, showed the presence of significant amounts of osteopontin in malignant ducts and the surrounding pancreatic acini [106]. Further studies suggested that nicotine contributes to PDA metastasis by inducing MMP9 and VEGF expression and osteopontin mediated these effects [107]. An osteopontin isoform, OPNc, is selectively inducible by nicotine and is highly expressed in PDA tissues from smokers which induced the expression of monocyle chemoattractant protein (MCP-1) indicating a proinflammatory role of nicotine [108]. Altogether, these results suggest that nicotine plays a key role in the regulation of the complex cellular cascades that modulate cell adhesion, invasion, and migration leading to metastasis.

## 7. Discussion and Conclusions

Tobacco smoking is a well-documented risk factor for many cancers. As summarized in Figure 1, nicotine, the principal addictive component of tobacco smoke, as well as other nitrosamines have been found to act through nAChRs on nonneuronal cells to facilitate tumor growth, angiogenesis, metastasis, survival, and chemoresistance by regulating diverse signaling pathways. Binding of agonist to nAChR facilitates the complex formation between the receptor, scaffolding protein $\beta$-arrestin and tyrosine kinase Src. Activation of Src was found to be important for cancer as well as endothelial cell proliferation and angiogenic tube formation *in vitro*. Proliferative effect of nAChR-activation was also supported by indirect stimulation of $\beta$-adrenergic receptor ($\beta$-AR) signaling. Further, chemotherapy-induced apoptosis was found to be blocked by nicotine-induced survivin expression as well as NF-$\kappa$B activation. Activation of nAChR is also correlated with EMT-like changes and metastatic dissemination of primary tumor cells. Given the ability of nicotine to affect various aspects of tumor growth and metastasis, antagonists of nAChR signaling might be beneficial in controlling the growth and progression of tumors. Recently, alpha cobratoxin ($\alpha$-CbT) has been shown to block the growth of a variety of NSCLC and mesothelioma cell lines both *in vitro* and *in vivo* [109, 110]. The most striking effect of $\alpha$-CbT was its ability to effectively inhibit the metastatic potential of lung cancer cells transplanted into nude mice, indicating the possibility of using nAChR antagonists as adjuvant therapy in preventing metastatic spread. At the same time, the potential side effects of nAChR antagonists on the brain and central nervous system need to be investigated before using them as a viable drug for combating lung cancer. Moreover, the direct role of nicotine alone on several aspects of tumorigenesis raises the need to revisit the potential tumor promoting effects of nicotine-replacement therapy. Also, the modulation effects of secondhand smoke on nAChRs require detailed investigation in the future.

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*Review Article*

# From Smoking to Cancers: Novel Targets to Neuronal Nicotinic Acetylcholine Receptors

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Cigarette smoking bears a strong etiological association with many neovascularization-related diseases, including cancer, cardiovascular disease, and age-related macular degeneration. Cigarette smoke is a complex mixture of many compounds, including nicotine, which is the major active and addictive component of tobacco. Nicotine and its specific metabolized carcinogens directly bind to nicotinic acetylcholine receptors (nAChRs) on cell membranes and trigger the nAChR signal cascade. The nAChRs were originally thought to be ligand-gated ion channels that modulate physiological processes ranging from neurotransmission to cancer signaling. For several decades, the nAChRs served as a prototypic molecule for neurotransmitter receptors; however, they are now important therapeutic targets for various diseases, including Alzheimer's and Parkinson's diseases, schizophrenia, and even cancer. This paper describes recent advances in our understanding of the assembly, activity, and biological functions of nicotinic receptors, as well as developments in the therapeutic application of nicotinic receptor ligands.

## 1. Introduction

The impact of tobacco use on mortality and morbidity is well known. As far back as 1982, the Surgeon General of the United States Public Health Service has concluded that cigarette smoking is the major single cause of cancer mortality in the United States. Recently, the World Health Organization (WHO) reported in 2010 that almost one billion people and 250 million women are daily smokers. The tobacco epidemic kills 5.4 million people in average per year from lung cancer, heart disease, and other illnesses, and approximately 650,000 of these deaths are caused by second-hand smoke. If this smoking trend continues, there will be more than 8 million deaths every year, with more than 80% of tobacco-related deaths in developing countries by 2030. Consequently, tobacco will kill a billion people due to smoking-related disease during this century, with tobacco use-related cancers being one of the main causes of death.

Tobacco use is by far the most widespread factor causing exposure to known carcinogens and death from cancer and is therefore a model for understanding mechanisms of cancer induction. A causal relationship was reported between active smoking and cardiovascular diseases, respiratory diseases, reproductive disorders, and several types of cancers, including cancers of the lung, bladder, cervix, esophagus, kidney, larynx, mouth, pancreas, stomach, and leukemia [1]. Although it might seem obvious that carcinogens associated with the use of tobacco products have caused numerous cancers, the effects of cancer genes, protein complexes, cellular circuitry, and signal transduction pathways are often overlooked.

According to the report from the International Agency for Research on Cancer in 2010, cigarette smoke contains a diverse array of 4,000 chemicals, 250 of which are known to be harmful, and more than 60 known carcinogens have been detected in mainstream cigarette smoke, and most of the same carcinogens are also present in second-hand smoke. The most potent of these carcinogens are polycyclic aromatic hydrocarbons and nicotine-specific metabolites, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

and N-nitrosonornicotine (NNN). These nitrosamines form DNA adducts cause mutations that lead to cancer [2]. DNA adducts have been proposed as potential markers of exposure to tobacco carcinogens, and these markers may help provide an integrated measure of carcinogen exposure relevant to individual cancer risk assessment. The adduct levels are generally higher in lung tissues of smokers than those of nonsmokers while studies using blood DNA have produced mixed results. In the following sections, we review evidence showing how nicotine or nicotine-specific metabolic nitrosamines, NNK or NNN, promote cancer development through the physical interaction with nicotinic acetylcholine receptors (nAChRs).

## 2. Genomewide Association of nAChRs with Lung Cancer

Many studies have pointed out that the binding of exogenous nicotine, NNK, NNN, and acetylcholine to nAChRs, respectively, will stimulate the growth of both small cell lung carcinomas (SCLCs) and nonsmall cell lung carcinomas (NSCLCs) [3]. Two similar studies also showed that the autocrine interaction of acetylcholine (Ach) and estrogen with the nAChR will stimulate SCLC and breast cancer cell proliferation [4–6]. To identify genetic factors involved in smoking-mediated cancer risk, a genomewide association study of 317, 139 single-nucleotide polymorphisms was recently performed using DNA from 1,989 lung cancer patients and 2,625 control subjects from six central European countries [7]. A locus in the 15q25 chromosome region was found to be strongly associated with lung cancer [8]. Interestingly, this region contains several genes, including three nAChR subunits (*CHRNA5*, *CHRNA3*, and *CHRNB4,* encoding the $\alpha 5$, $\alpha 3$, and $\beta 4$ subunits, resp.) that are predominantly expressed in neurons and other tissues (particularly alveolar epithelial cells, pulmonary neuroendocrine cells, and lung cancer cell lines) [7, 9, 10]. Previous studies have also suggested that *N′-nitrosonornicotine* and nitrosamines may facilitate neoplastic transformation by stimulating angiogenesis and tumor growth mediated through their interaction with nicotinic acetylcholine receptors [11, 12]. The activation of these receptors can also be inhibited by nicotine receptor antagonists, which confirms that nAChRs play important roles in disease development and implies possible chemoprevention opportunities for lung cancer [13]. Therefore, further analyses of multiple diverse populations will be required to confirm this locus and to identify additional lung cancer susceptibility.

## 3. Nicotinic Acetylcholine Receptor Structure

The nicotinic acetylcholine receptors (nAChRs) belong to the superfamily of the Cys-loop ligand-gated ion channels (LGICs), which also include the GABA, glycine, and 5-HT3 receptors. They are formed by the assembly of five transmembrane subunits selected from a pool of 17 homologous polypeptides ($\alpha 1$–10, $\beta 1$–4, $\gamma$, $\delta$, and $\varepsilon$). There are many nAChR subtypes, each consisting of a specific combination of subunits, which mediate diverse physiological functions. These receptors are widely expressed in the central nervous system, and, in the periphery, they mediate synaptic transmission at neuromuscular junctions and ganglia. Recently, the cDNAs for all types of nAChR subunits have been cloned from neuronal and nonneuronal cells, such as keratinocytes, epithelia, and macrophages, which encompass the main domains of the ligand-binding sites.

Based on the different ligand-binding properties of these nAChRs, the nAChRs are divided into two main classes: (1) the $\alpha$-bungarotoxin-($\alpha$-Bgtx-) binding nAChRs, which are mainly homopentamers of $\alpha 7$, $\alpha 8$, or $\alpha 9$ subunits; (2) nAChRs, which do not bind $\alpha$-Bgtx but consist of the $\alpha 2$–$\alpha 6$ and $\beta 2$–$\beta 4$ subunits, exist only as heteropentamers and bind agonists with high affinity [14]. The presence of a certain subunit can affect the localization, biophysical, functional, and pharmacological properties of the nAChRs, as well as the regulation of the expression of the nAChR subtype at the developmental or adult stage in some specific cells. The absence of a subunit may lead to the compensatory upregulation of other subtypes [15].

Because nAChR subunits exhibit a high degree of evolutionary conservation, studies of high-resolution X-ray crystallographic and electron microscopic analyses of proteins related to nAChRs have provided considerable insight into how structure imparts functional similarities and differences among all nAChRs.

Multiple nAChR subunit compositions are expressed in the central and peripheral nervous system, but the most represented receptors are $\alpha 4\beta 2$ and $\alpha 7$ in the brain and $\alpha 3\beta 4$ in the peripheral nervous system. In these nAChRs, $\alpha 4\beta 2$-composed nAChRs have the highest affinity to nicotine [16, 17]. In addition, only the $\alpha 4$ and $\beta 2$ subunits are found on GABA-Aergic neurons [18]. Another study pointed out that $\alpha 4\beta 2$ levels can be upregulated by proinflammation cytokines, such as TNF-$\alpha$ [19] through p38-MAPK signaling pathways. This important discovery reveals the complexity of the interaction network between nAChRs and the inflammation factors. By contrast, compared to $\alpha 4\beta 2$ nAChR, $\alpha 7$ homopentameric nAChR is the most well-known and investigated type of nAChR. Receptors composed of $\alpha 7$ subunits are known to desensitize rapidly and to have a high $Ca2^{+}:Na^{+}$ permeability ratio that exceeds that of the glutamate NMDA receptor [20–22] and the 3-4 : 1 ratio of most other nAChRs. The signaling pathway encourages scientists to look further into carcinogenetic mechanisms underlying $\alpha 7$-nAChRs-related lung [23], bladder [24], and colon cancers [25], as well as $\alpha 9$-nAChRs in breast cancers [26–28]. In fact, some receptors (such as $\alpha 7$, $\alpha 9$, and $\alpha 10$) have highly specialized functions including those pertaining to the regulation of signaling mechanisms used by sensory epithelia and other nonneuronal cell types [29].

## 4. nAChR Signaling Pathways

Cigarette smoking has a strong etiological association with the development and progression of several types of cancers, cardiovascular disease, diabetic retinopathy, and age-related macular degeneration. Nicotine is the major active and addictive component of cigarette smoke. Previous studies

demonstrated that the average plasma nicotine concentration of active smokers is about 100 nM to 1 $\mu$M [30, 31]. In addition to active cigarette smoking, exposure to second-hand smoke is another mode of nicotine exposure [32]. When the biological levels of nicotine associated with second-hand smoke exposure were measured, a positive correlation between second-hand smoke exposure and concentrations of nicotine in the body was found. To date, it is well known that the specific nicotine-metabolized, tobacco-specific carcinogenic nitrosamines NNK and NNN are strong mutagens associated with several cancers, including lung, bladder, colon, and breast cancers [33–38]. Through binding of several ligands and nAChRs, signaling transductions are able to activate and promote cell proliferation, migration, and metastasis in cancer cells.

As the nAChRs are ligand-gated cationic channels, their different subtypes, such as neuronal nAChRs, are differentially permeable to calcium ions [39, 40]. The calcium permeability of homomeric receptors is significantly higher than heteromeric nAChRs [39, 40]. In particular, the $\alpha$7-containing nAChRs are generally considered to be the most permeable receptors to calcium, and their activation can raise cytoplasmic calcium levels and trigger a series of calcium-dependent intracellular processes [39, 40]. Recent studies have demonstrated the presence of nAChRs in several nonneuronal, nonexcitable cells, including bronchial epithelium, endothelial cells, keratinocytes, immune cells, and vascular smooth muscle cells [15, 41, 42]. The presence of these receptors in nonneuronal cells seems to suggest that they have distinct functions well beyond neurotransmission [43–49].

Several convergent studies have indicated that the $\alpha$7-nAChRs primarily mediate endothelial cell proliferation, invasion, and angiogenesis [50–55]. The presence of $\alpha$7-nAChR inhibitors like methyllycaconitine (MLA) and $\alpha$-bungarotoxin could reverse the proangiogenic effects of nicotine. However, it must be noted that both $\alpha$-bungarotoxin and MLA also bind with high affinity to $\alpha$9-nAChR. Therefore, there may be partial involvement of $\alpha$9-nAChR in the proangiogenic effects of nicotine [56]. The involvement of nAChR subunits in nicotine-induced angiogenesis was further verified by siRNA techniques.

In general, nicotine induced cell proliferation, angiogenesis, migration, and apoptosis through nAChR-associated downstream signal transduction such as MAP Kinase, PI3-kinase/Akt, NF-$\kappa$B, and $\beta$-arrestin pathways [50–53, 57–60]. Through the above signal transduction, it is found that in nonneuronal tissues, nicotine induces the secretion of growth factors such as $\beta$FGF, TGF$\alpha$, VEGF, PDGF [61], and the upregulation of the calpain family of proteins [62], COX-2 and VEGFR-2 [63]. Most intriguingly, both VEGF- and $\beta$FGF-induced human microvascular endothelial cell (HMVEC) migration and angiogenesis require nAChR activation [64].

Mechanistically, nicotine has been shown to induce activation of NF-$\kappa$B through the MAP kinase and PI3K/AKT signaling pathways, which promote survival, proliferation, and angiogenesis of endothelial cells [65]. Further study showed pharmacological dissection of nicotine's influence on cell cycle progression, apoptosis, and differentiation [43], and this indicates that $\alpha$7-nAChRs expressed in keratinocytes are important. In addition, large-cell carcinoma, squamous-cell carcinoma, adenocarcinoma of small airway, and alveolar type II cell of origin, as well as immortalized large- and small-airway epithelial cells all confirmed that nicotine and NNK activate the PI3K-Akt pathway and NF-$\kappa$B, resulting in stimulation of proliferation and inhibition of chemotherapy-induced apoptosis [66, 67]. Recently, a study also demonstrated that AKT survival signals play an important role in the nicotine-mediated carcinogenic process in human breast cancer cells [28].

In addition, $\beta$-arrestin-1 and Src kinase also appear to be the key players in mediating the mitogenic effects of nicotine. The Src family of protein tyrosine kinases has been found to be a critical component of multiple receptor-mediated signaling pathways that regulate proliferation, survival, metastasis, and angiogenesis. Additionally, nicotine also promotes cancer cell invasion by inducing matrix metalloproteinases 2 and 9, as well as the expression of plasminogen activators (urokinase-type plasminogen activator and its receptor) through COX-2 and VEGFR regulation [63].

Taken together, nicotine promotes cell proliferation and tumor angiogenesis via the stimulation of nAChRs. Nicotinic receptor antagonists, such as mecamylamine and $\alpha$-bungarotoxin, demonstrate potent therapeutic application. Therefore, the development of specific, potent nAChR analogs and antagonists could provide novel approaches for the treatment of neovascularization-related diseases including cancer, cardiovascular disease, and macular degeneration.

## 5. Smoking, nAChRs, and Cancer

Cigarette smoking bears a strong etiological association with cancers. To the best of our knowledge, smoking-induced transformation can be abstracted into two aspects: (1) among the mixture of cigarette smoking compounds, NNK and NNN play the role of initiators in carcinogenesis (indirect-acting carcinogens, the most important tumorigenesis model in lung and bladder cancers). In contrast, nicotine has been demonstrated as a cocarcinogenic factor by playing a promoter role of carcinogenesis in tobacco replacement therapies [68]. Nicotine and NNK are considered to be carcinogens that react to DNA, and most reports have proposed that the chemical properties of the resulting DNA adducts can cause diverse genetic changes known to exist in human cancers [69–71]. (2) Nicotine, NNN or NNK have strong abilities to upregulate nAChR expressions which promote signals cascade, all these events result in a strong feedback loop and cause enhancement of cancer cell proliferation, migration, and metastasis. Therefore, understanding the functional diversity of the nAChR in each tissue could offer useful and abundant prospects for the designing of the novel cancer therapeutics stratagem.

Since the brain is the best organ to characterize the role of nAChR in the regulation of the neurotransmitter acetylcholine [72], the interaction of nicotine with nAChR

subunits in the brain provides the basis for nicotine addiction. For decades, nAChRs were generally believed to exist only in the nervous system (neuronal nAChRs) and at neuromuscular junctions (muscle nAChRs). However, in the past 20 years, increasing studies have shown that nAChRs can also be expressed in oral [34], mechanosensory hair [73], and airway epithelium cells [74, 75], where they play different roles in normal cell development and function. Furthermore, recent studies have also shown evidence that nAChRs and their physiological ligands such as choline and acetylcholine are universally expressed in mammalian and, more importantly, in cancer cells [24–26, 76–80]. The first study that implicated nAChRs in cancer growth regulation was reported in 1989 [81], and in the following decades, many studies indicated that nAChRs are the key molecular and central regulators of a complex network of stimulatory and inhibitory neurotransmitters that govern the synthesis and release of growth [23, 26, 82, 83], angiogenic [58], metastasis [25], and even apoptosis [84–86] in cancer cells microenvironment. In addition, the nAChRs are also found to trigger intracellular signaling pathways in a cell-type-specific manner.

The expression of nAChRs in mammalian cells and their diverse regulatory functions suggest that the modulation of these receptors, owing to chronic exposure to tobacco constituents or other environmental and lifestyle factors, contribute to the development of cancer [1]. This hypothesis was supported by the discovery that the tobacco-specific carcinogenic nitrosamines NNK, and NNN are agonists of $\alpha$7-nAchR and the heteromeric $\alpha\beta$nAChRs, respectively [87]; both these nitrosamines cause lung cancer in laboratory animals [88]. The affinity of NNK for $\alpha$7-nAChR was found to be about 1,300-fold higher than for nicotine, whereas the affinity of NNN for the heteromeric $\alpha\beta$nAChRs was about 5,000-fold higher [34, 87]. Because of their high affinity for nAChRs, NNK and NNN, rather than nicotine, might be the actual ligands for nAChRs in the context of smoking tobacco. Therefore, many of the addictive, neuropsychological, and cancer-stimulating effects from smoking that are currently attributed to nicotine are probably caused by these nitrosamines. In support of this hypothesis, a study displayed that the binding of NNK to $\alpha$7-nAChR causes an influx of $Ca^{2+}$ into lung cells, and the resulting membrane depolarization activated voltage-gated $Ca^{2+}$ channels [49]. These eventually upregulated nAChRs expression [27, 89]. These data demonstrated a strong positive feedback loop associated with nAChR signaling that eventually causes normal cells to step into precancerous phase of transformation. Although all nAChRs are cation channels, they regulate diverse functions in a cell-type-specific manner. This functional diversity is also reflected in cancers of different cellular origins. In the following sections, the two latest and most important nAChR-induced cancer formation models will be illustrated.

## 6. $\alpha$7-nAChRs and Lung Cancer

The presence of nAChRs in lung cancer cell lines has been well investigated since 1989-1990; the first report suggested that nicotine and NNK bound to nAChRs would stimulate the proliferation of human small cell lung cancer (SCLC) cells through autonomic nervous-system-dependent regulation of lung cancer cells [81, 90]. This study was then reviewed by Maneckjee and Minna in 1990 and 1994 who demonstrated a nicotine-induced reversal of apoptosis in response to opioids in SCLC and NSCLC cell lines [91, 92]. Another laboratory discovered that nicotine affects the proliferation of human SCLC cell lines by stimulating the release of serotonin, which acts as an autocrine growth factor in these cells [93]. In turn, these findings led to the hypothesis that human airway epithelial cells express all of the components required to synthesize and secrete members of the acetylcholine family and nAChR subtypes.

Nicotine exposure induces the augmented expression of $\alpha$7-nAChRs, which causes an influx of $Ca^{2+}$ and activates downstream signals, such as protein kinase C, Raf-1, extracellular-signal regulated kinase (ERK) 1/2, and c-Myc, leading to increases in cell proliferation, cancer cell migration, metastasis, or the inhibition of apoptosis. West and colleagues [66] suggested that redundant Akt activation by nicotine and its carcinogen derivative NNK could contribute to tobacco-related carcinogenesis in nonimmortalized human airway epithelial cells. In this study, normal human bronchial epithelial (NHBE) cell was forced to be transformed through nicotinic activation of Akt which alters epithelial cell growth characteristics. Dysregulated NHBE growth after nicotine administration is consistent with *in vivo* observations of active smokers in which increased proliferative indices were seen when compared with former smokers. Protection from prolonged serum-deprivation-induced apoptosis, conferred by nicotine was attenuated by LY294002 or by DH$\beta$E, and protection conferred by NNK, was attenuated by LY294002 or by $\alpha$-BTX [66]. This study showed that, in addition to promoting cellular survival or transformation process, nAChR activation from nicotine or NNK-induced Akt signal is required for diminishing contact inhibition and cellular dependence on exogenous growth factors or extracellular matrix. It revealed that abundant $\alpha$7-nAChR expression in human cancer cells could be selectively attenuated by specific antagonists. Recently, Schuller [94] also proved that NNK interacts with $\alpha$7 nAChRs, resulting in the development of lung cancer. The signals involved in normal cell transformation might be due to a significant reversible upregulation of the $\alpha$1, $\alpha$5, and $\alpha$7 subunits in human bronchial epithelial cells, when these cells were exposed to nicotine (100 nM) *in vitro* for 72 hours. Since studies have shown that $\alpha$7 is the main nAChR subunit that mediates the proliferative effects of nicotine in lung cancer cells [33, 95–99], $\alpha$7-nAChR might be a valuable molecular target specifically for lung cancer therapy [100–102].

## 7. $\alpha$9-nAChRs and Breast Cancer

The expression of estrogen receptors by breast cancer cells has provided a therapeutic target by using estrogen receptor antagonists, but their use contributes to an unfortunate stimulation of breast cancer development through the pharmacological use of estrogen, the ligand for the estrogen

receptor. Estrogen was recently found to differentially modulate nAChR subtype [5], and the expression of nAChR by breast cancers may similarly provide a new target for breast cancer therapies, whereas nicotine, a ligand for nAChR, was found to have stimulated breast cancer growth.

The $\alpha$9-nAChR is a known homopentamer that plays a central role in coordinating keratinocyte adhesion and motility during wound healing [4]. Lee et al. [26] showed that $\alpha$9-nAChRs were found to be ubiquitously expressed in many epithelial, lung, and breast cancer cell lines, and that most of the same cell lines also expressed $\alpha$5- and $\alpha$10-nAChRs [26]. The $\alpha$9-nAChRs were present in primary tumors and nonmalignant breast tissue obtained from patients; however, breast cancer cells had increased $\alpha$9-nAChR expression compared with the surrounding normal tissues. Lee et al. used MDA-MB-231 breast cancer cells, in which $\alpha$9-nAChR expression had been silenced, to show that lowering $\alpha$9-nAChR expression would reduce proliferation and tumorigenic potential in both *in vitro* and *in vivo* assays. Cells with inducible $\alpha$9-nAChR gene expression were also generated from normal breast epithelial cells (MCF-10A) that were transformed by nicotine or NNK treatments, and experiments showed that increased $\alpha$9-nAChR expression *in vitro* enhanced proliferation and colony formation. Likewise, mice that were subcutaneously injected with nicotine-transformed MCF-10A cells that inducibly expressed increased levels of $\alpha$9-nAChRs showed enhanced tumor xenograft volumes when exposed to nicotine. Several studies have reported that nicotine decreases the cytotoxicity of doxorubicin, promotes migration via a signaling cascade involving protein kinase C and cdc42, and induces the proliferation, invasion, and epithelial-mesenchymal transition of breast cancer cells [103–105]. These studies provided evidence that nAChR, more specifically $\alpha$9-nAChR, might play a major role in breast carcinogenesis, just as $\alpha$7-nAChR is often associated with lung cancer [66], which further supports epidemiological studies that have revealed an association between breast cancer and exposure to cigarette smoke [106]. In conclusion, all the above demonstrated that $\alpha$9-nAChRs expression knockdown can indeed inhibit breast cancer cell growth, whereas overexpression of $\alpha$9-nAChRs, accompanied with long-term treatment nicotine, causes normal breast epithelial cell transformation both *in vitro* and *in viv*o experimental studies.

## 8. Developing Drugs Targeted at nAChR

*8.1. nAChRs Agonist.* Recent studies have shown that nicotine is not only a harmful product in cigarettes, but it is also a therapeutic nAChRs stimulator that enhances wound healing in preclinical models [53, 58, 107, 108]. Notably, these studies were conducted in animal models, and no side effects of nAChR agonists or antagonists were reported. Several neurological diseases associated with aging have been linked to reduced angiogenesis in the brain, and changes in the levels of nAChR in vascular-related cells in Alzheimer's disease [109, 110], and this suggests that there could be a role for a nicotine-based therapy in neurological disorders.

Many of the studied, clinically used drugs that target nAChRs are administered for months, resulting in long-term changes in receptor properties and/or number. Accordingly, these drugs can be divided into two categories: $\alpha$7-nAChR or non-$\alpha$7-nAChR target agents. There are many potential drugs targeting nAChRs, and most of them are agonists and can be applied to treatment of various nervous-system disorders. For example, GTS-21, TC-5619, or EVP-6124 can be used for schizophrenia therapies [111]. The major target disease for a cognition enhancer is Alzheimer's disease. In Alzheimer's brain tissue, cortical nAChRs ($\alpha4\beta2$) are markedly reduced (>80%), reflecting the cholinergic deficits associated with Alzheimer's disease [112]. Pilot trials using nicotine patches have demonstrated improved attention in Alzheimer's disease patients [113]. Interestingly, pharmacoepidemiological studies have shown a reduced incidence of Alzheimer's disease in populations of individuals who have previously smoked [114]. The potential protective effects of (−)-nicotine in this neurodegenerative disease may be related to neuroprotective properties observed with nicotine and other nAChR activators in *in vitro* and *in vivo* experimental studies. To our knowledge, Alzheimer's disease-specific therapies are mainly agonists of $\alpha$7-nAChR. For example, SSR-180711, MEM-3454/R-3487, MEM-63908/R-4996, AZD-0328, and S-24795 are used. The $\alpha4\beta2$-nAChR agonists are TC-1734 and S-38232; these drugs have shown promises in preclinical cognition models [111]. Other related drugs that act on the $\alpha4\beta2$-nAChR can also be applied to smoking cessation, attention-deficit hyperactivity disorder, cognitive dysfunction, and depression [111].

*8.2. nAChRs Antagonist.* Neurotoxins are commonly used to distinguish between neuronal nAChR receptor subunit combinations [115, 116]. The neurotoxins lophotoxin, neosurugatoxin, erysodine, $\alpha$-BgT, and the alkaloids DH$\beta$E are competitive nAChR antagonists that display selectivity for $\beta$2-containing nAChRs, particularly the $\alpha4\beta2$ subtype [117]. The latest study implied that nAChR antagonists can be used for anticancer drugs. While the $\alpha$7-nAChRs are overexpressed in small-cell lung carcinoma in smokers [118], *in vitro* experiments have suggested that the malignant growth can be ceased using snake neurotoxins ($\alpha$-neurotoxins) or snail conotoxins ($\alpha$-conotoxins), and these have been used for the isolation and biochemical characterization of nAChRs because they are competitive antagonists of the nAChR [119]. The presence of $\alpha$7-nAChR inhibitors, such as methyllycaconitine (MLA) and $\alpha$-bungarotoxin, was found to have reversed the proangiogenic effects of nicotine during cancer development process [50–52, 54]. Russo and colleagues demonstrated that several natural compounds significantly inhibited NSCLC cell proliferation or tumor growth by inhibition of $\alpha$7-nAChR expression. These data determined a significant reduction of tumor growth in nude mice orthotopically engrafted with A549-luciferase cells (4.6% of living cells versus 31% in untreated mice). The specific $\alpha$7-nAChR antagonists can undergo both induction of apoptosis protein (activates caspases 3, 9, 2, P53, and Bad) and reduction of survival signaling (activates PI3K-Akt, MAPK, and NF-$\kappa$B pathways) in *in vitro* and *in vivo*

experiments. These data suggested that $\alpha$7-nAChR-targeted chemicals form a promising prospective in anticancer drug development. However, it must be noted that both $\alpha$-bungarotoxin and MLA also bind in high affinity to $\alpha$9-nAChR. Therefore, there may be partial involvement of $\alpha$9-nAChR in the proangiogenic effects of nicotine [120, 121].

Recently, the involvement of $\alpha$9-nAChR in pain has been suggested by a number of experimental observations, and the administration of nAChR agonists reduces pain-related behaviors in several studies [122–124]. Virus-mediated overexpression of the $\alpha$9-nAChR subtype was specifically found in breast cancer tumors [26]. Rather than using competitive nAChR inhibitors, nature compounds were investigated and shown to have inhibited cancer cell proliferation. For example, a very low concentration of garcinol (1 $\mu$M) from the edible fruit *Garcinia indica* inhibited nicotine-induced breast cancer cell proliferation through the downregulation of $\alpha$9-nAChR and cyclin D3 expression [27]. Other natural compounds, such as *luteolin* and *quercetin*, have also inhibited human breast cancer cell proliferation through the downregulation of cell surface $\alpha$9-nAChR subunit expression in human breast cancer cells, and the combined treatment of cells with *luteolin* and *quercetin* synergistically inhibited AKT activation [28]. In another study, Tu et al. found that estradiol- and nicotine-induced $\alpha$9-nAChR protein expression was blocked by *epigallocatechin-3-gallate* (EGCG) [125]. These findings suggested a possible chemopreventive ability of EGCG through the inhibition of estrogen- or nicotine-induced $\alpha$9-nAChR protein expression, which is known to confer smoking-mediated breast tumorigenesis. All of these findings have provided molecular evidence for the possible chemopreventive or chemotherapeutic ability of smoking-mediated breast tumorigenesis. As always, a balance of regulating nAChR activity must be maintained between limiting pathological angiogenesis and causing potential toxicity to patients.

## 9. Conclusion

Epidemiological and experimental studies targeting nAChRs have clearly established that tobacco products cause cancers of various types. An improvement of understanding towards any relevant carcinogenic mechanisms will lead to new approaches for cancer prevention. Over the past two decades, several valuable tobacco carcinogen biomarkers have been discovered, which increases our insight into the mechanism of cancer induction. The multiple tumor-promoting effects caused by cigarette smoke and the carcinogens and toxicants in it must be targeted. The ideal drug to target these effects must have minimal toxicity in animal models and humans, which might be achievable through using naturally occurring compounds in doses no greater than those present in common foods, such as vegetables, to maintain homeostasis in the human body. At present, the majority of compounds under investigation are either agonists or partial agonists. Given the negative effects of nicotine on the immune system function, receptor subtype-selective antagonists might also be beneficial as therapeutic agents. The presence of nAChRs in tissues, in addition to the central and peripheral nervous systems, for example, immune system, gastrointestinal tract, lung, breast, and bladder, could offer additional therapeutic targets for receptor subtype-selective nAChR ligands when these become available.

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*Review Article*

# NNK-Induced Lung Tumors: A Review of Animal Model

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The incidence of lung adenocarcinoma has been remarkably increasing in recent years due to the introduction of filter cigarettes and secondary-hand smoking because the people are more exposed to higher amounts of nitrogen oxides, especially 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone(NNK), which is widely applied in animal model of lung tumors. In NNK-induced lung tumors, genetic mutation, chromosome instability, gene methylation, and activation of oncogenes have been found so as to disrupt the expression profiles of some proteins or enzymes in various cellular signal pathways. Transgenic animal with specific alteration of lung cancer-related molecules have also been introduced to clarify the molecular mechanisms of NNK in the pathogenesis and development of lung tumors. Based on these animal models, many antioxidant ingredients and antitumor chemotherapeutic agents have been proved to suppress the NNK-induced lung carcinogenesis. In the future, it is necessary to delineate the most potent biomarkers of NNK-induced lung tumorigenesis, and to develop efficient methods to fight against NNK-associated lung cancer using animal models.

## 1. Introduction

Throughout the spectrum of cancers worldwide, lung cancer claims the lives of over one million people worldwide each year and is one of the most common and lethal cancers of men and women in North America, Europe, and East Asia although current strategies in the treatment of lung cancer including surgery, radiation therapy, chemotherapy, and targeted biological therapies have slightly generated an increase in the 5-year survival rate for all stages combined [1]. The secular trend in lung cancer histology indicates that the proportion or incidence of lung adenocarcinoma has been increasing markedly over the past two decades, surpassing the squamous cell carcinoma as the most common histological subtype of lung cancer in many countries, which is partly due to the introduction of filter cigarettes and secondary-smoking because the people are more exposed to higher amounts of nitrogen oxides, nitrosated compounds, and lung-specific smoke carcinogens [1–3].

Epidemiological and laboratory evidences demonstrate a strong etiological association with smoking, which contains volatile N-nitrosamines such as N-nitrosodimethylamine, N-nitrosopyrrolidine as well as tobacco-specific N-nitrosamines such as N′-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Although N′-nitrosonornicotine causes tumors of the oesophagus and nasal cavity in rats, NNK reproducibly induces pulmonary adenocarcinomas (PAC) in laboratory rodents, including rats, mice, hamsters, and ferrets, which therefore has been classified as a human lung carcinogen by the International Agency for Research on Cancer working group. Significant incidences of tumors occurred in the lungs of strain A/J progeny (24 wk) and in the livers of male C3B6F1 and Swiss progeny (72 wk) after NNK treatment [4]. NNK is known to be activated in the lung via $\alpha$-carbon hydroxylation by cytochrome P450 (CYP), hemoglobin, and lipoxygenases (LOX) [5]. The production of methylating and pyridyloxobutylating agents that attack DNA and cause the genetic changes is known to be associated with self sufficiency in growth signals, evasion of apoptosis, insensitivity to antigrowth signals, sustained angiogenesis, tissue invasion and metastasis, and limitless replicative potential.

With lung carcinogenesis models, it may be helpful to gain insights into basic biology of lung tumors, find out markers for early diagnosis, and validate antilung cancer

prevention and therapies. Ferrets exposed to both NNK and smoke developed preneoplastic lesions (squamous metaplasia, dysplasia, and atypical adenomatous hyperplasia) with complex growth patterns and further exposure will cause squamous cell carcinoma, adenosquamous carcinoma, and adenocarcinoma [6, 7]. In A/J mice, 14-week NNK treatment can cause pulmonary hyperplasias along the alveolar septa, in which the proliferating cells showed cuboidal shape, lamellar bodies, and centrally localized ovoid nuclei as type II pneumocytes. From 34 to 42 wks after treatment, progression to neoplasia was characterized by a declined hyperplasias and an increased adenoma. Carcinomas appeared to increase in frequency 34 wks after NNK treatment and comprised more than 50% of the pulmonary lesions by 54 wks. The growth pattern of carcinomas began to change from solid to mixed (solid and papillary) 42 wks after NNK treatment [8].

## 2. Mechanisms of NNK-Induced Lung Carcinogenesis

The establishment of genetic and epigenetic alteration followed by gene expression profiling is of great use and help to clarify molecular mechanisms of NNK-induced lung tumorigenesis. In experimental model, NNK could cause Adrb2 SNP mutation of Syrian golden hamsters, and K-ras mutation in codon 12 of the A/J mice [9–11]. Bacterial artificial chromosome array-based comparative genomic hybridization indicated that the gains on chromosomes 6 and 8, and losses on chromosomes 11 and 14, were more common in NNK-induced tumors and the changes on chromosomes 8, 11, 12, and 14 were positively associated with the degree of chromosome instability [12, 13]. The methylation of the retinoic acid receptor (RAR)-$\beta$ and death-associated protein kinase gene was also detected in preneoplastic hyperplasias or adenocarcinoma induced by NNK treatment [14–16].

NNK administration reduced the miR-126* expression targeting CYP2A3 in rat lungs, but induced CYP2A3 expression [17]. The 14-3-3 isoforms ($\theta$, $\xi$, and $\sigma$) and annexin A5 were significantly downregulated in NNK-induced pulmonary adenocarcinogenesis of A/J mice according to 2D-electrophoresis [18]. Immunohistochemically, NNK induced preneoplastic lesions in lungs, including alveolar hyperplasia and atypical dysplasia with COX-2 and PCNA overexpression [19]. In lung adenocarcinoma of hamsters, the overexpression of PKA, cAMP, CREB and phosphorylated CREB in $\beta$2-adrenergic receptor pathway and EGFR-specific phosphorylated tyrosine kinase, Raf-1 and ERK1/2 and their phosphorylated forms in EGFR pathway were observed [20]. Regarding the control of cell cycle, there was an upregulated expression of cyclin D1 and cdk4, but downregulated Rb expression in NNK-induced lung adenomas and adenocarcinomas [21]. Exposure of NNK to hamsters and mice led to the decreased expression of Clara cell 10-kDa protein (CC10) which subsequently enhances the induction of anchorage-independent growth in response to NNK [22]. The NNK treatment enhanced the expression of fatty acid synthase, transketolase, pulmonary surfactant-associated protein C, L-plastin, annexin A1, and haptoglobin, but the expression of transferrin, $\alpha$-1-antitrypsin, and apolipoprotein A-1 decreased [23]. The NNK-mediated expression of protein and RNA in mouse lung tumors will provide more information and clues about markers of NNK-induced tumroigenesis and targets for antipreventive agents in NNK-related lung cancer.

## 3. Transgenic Animal Model of NNK-Mediated Lung Carcinoma

Transgenic models have the potential to play an important role in identification of potential human carcinogens and clarify the molecular mechanisms of carcinogens in the pathogenesis and development of malignancies. Actually, many transgenic and knockout mice have been applied to investigate the NNK-induced lung carcinogenesis.

When transgenic mice that overexpress HGF in the airway epithelium were exposed to NNK, they exhibited congestion in the alveolar spaces, excessive production of blood vessels, a convoluted pattern of airways with more number of lung tumors, and high tumor incidence, compared with control [24]. All NNK-treated SPC/myc transgenic mice showed bronchioloalveolar hyperplasia and adenocarcinoma formation [25]. The p53 mutation, on an A/J F1 background, were more susceptible to NNK and mice with a mutant p53 developed larger lung tumors, emphasizing the potential effects of a p53 mutation both on tumor initiation and progression [26]. NNK-treated mice expressing high levels of IGF-IR transgene developed larger tumors than the control mice [27].

Galectin-3, a $\beta$-galactoside-binding lectin is a multifunctional protein, which regulates cellular adhesion, proliferation, and apoptosis, and in turn contributes to tumorigenesis. We intraperitoneally administrated NNK into galectin-3 wild-type (gal3+/+) and knockout (gal3−/−) mice and found that the incidence of lung tumors was significantly lower in gal3−/− than gal3+/+ mice after 32 wks. Compared with gal3+/+ mice, pathway analysis of gene microarray indicated that galectin-3 upregulated carcinogenesis-related genes (e.g., B-cell receptor, ERK/MAPK, and PPAR signalings) in normal condition, and NNK-induced gene expression were associated with cellular growth (e.g., Wnt/$\beta$-catenin signaling) or immunological disease (e.g., EGF and PDGF signalings) in lung carcinogenesis regardless of galectin-3 status. The functions involved in NNK-induced PAC include cellular growth and proliferation and canonical pathways for Wnt/$\beta$-catenin signaling [28, 29].

Human methylguanine-DNA methyltransferase (MGMT) transgenic mice overexpressing $O^6$-alkylguanine-DNA alkyltransferase (AGT) in lung were crossbred with A/J mice for animal model of lung tumors. The MGMT transgenic mice had higher AGT activity, lower multiplicity, and smaller-sized lung tumors than the control mice after NNK treatment. Moreover, a reduction in K-ras mutations in lung tumors was found in the MGMT transgenic mice [30]. When MGMT−/− mice were crossed with a lacI-based transgenic reporter line, NNK-dependent lacI mutations

was more frequently observed in MGMT−/− tissues. The mutational spectra of NNK-treated MGMT−/− lungs revealed an increase in G:C to A:T changes accompanied by a shift from CpG to GpG sites [31]. 8-hydroxyguanine DNA glycosylase 1 (Ogg1) gene encodes an enzyme that repairs an oxidative DNA injury 8-oxoguanine (8-oxoG), whose deficiency results in the development of lung adenomas and preneoplastic atypical hypreplasias in knockout mice treated with NNK [32].

There was a NNK-dose-dependent increase in lung tumor size in PTEN+/−, compared with +/+ mice. Lung tumors from PTEN+/− mice had K-ras mutations, low PTEN expression, and Akt pathway activation [33]. Although mice with a knockout of G-protein coupled receptor 5A develop lung tumors after a long latent period, NNK treatment could hurry the development of lung tumors, exhibiting increased tumor incidence and multiplicity and a dramatic increase in lesion size [34]. The 4 eukaryotic initiation factor (ebp)1−/−/4ebp2−/− mice showed increased sensitivity to NNK-induced tumorigenesis, compared with the wild-type counterparts due to translational activation of genes governing angiogenesis, growth and proliferation and translational activation of CYP2A5 [35]. NNK exposure of CC1-knockout mice causes a significantly higher incidence of airway epithelial hyperplasia and lung adenomas with K-ras mutation, Fas ligand expression and MAPK/ERK phosphorylation increased than wild-type littermates [36].

The numbers of NNK-induced lung tumors and tumor multiplicity were reduced in the lung-NADPH-P450-reductase (Cpr)-null mice, relative to wild-type mice, which was correlated with lower lung O6-methylguanine adduct levels. Lung tumors in lung-Cpr-null mice were positive for CPR expression, indicating that the tumors did not originate from Cpr-null cells [37]. With the NNK treatment, the tumor multiplicity in angiotensin II type 2 receptor (AT2)-null mice was significantly smaller than that in wild-type mice [38].

## 4. Repressing of NNK-Induced Lung Carcinogenesis

The antioxidants (Selenium, $\beta$-carotene, N-acetylcysteine, and $\alpha$-tocopherol) from tea, plant, or vitamin can prevent lung carcinogenesis in an NNK-treated ferret model by preventing oxidative DNA damage, and increasing the levels of lung retinoic acid in the lung cancer induced by NNK. Chemotherapeutic agents also play a preventive role in the NNK-induced lung tumorigenesis due to the modification of disrupted signal pathways.

*4.1. Selenium.* 1,4-phenylenebis(methylene)selenocyanate (p-XSC) was highly effective to inhibit the initiation and postinitiation phase of lung tumorigenesis induced by NNK in A/J mice and reduce NNK-induced DNA methylation and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in the lung [39]. The levels of protein-bound:free GSH ratios and Cys ratios were significantly decreased, and total glutathione S-transferase (GST) enzyme activity, as well as GST-pi and GST-mu enzyme activities, glutathione peroxidase (GPX) activity were significantly induced in p-XSC-treated mice after NNK treatment. These results suggest that p-XSC inhibits tumor formation partially by protecting against oxidative damage [40]. Additionally, p-XSC was shown to significantly inhibit formation of O6-methylguanine and 7-methylguanine in the mouse lungs treated with NNK, indicating its inhibitory role in DNA methylation [41]. Additionally, 2-oxo-selenazolidine-4(R)-carboxylic acids and selenocystine significantly reduced lung adenoma multiplicity in NNK-treated mice with hepatic selenium levels elevated [42].

*4.2. Tea.* The inhibitory effect of tea on lung carcinogenesis has been attributed to its major ingredients, such as polyphenolic compound, epigallocatechin gallate (EGCG), caffeine, thearubigins, and theaflavins because tea inhibited the formation of reactive oxygen species and radicals, and induced CYP1A1, 1A2 and 2B1, and glucuronosyl transferase [43]. In NNK-induced lung tumors, tea treatment inhibited angiogenesis, as indicated by the lower microvessel density and enhanced the apoptosis index labeled by TUNEL [44]. The levels of 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, were significantly suppressed in NNK-induced mice treated with green tea or EGCG. The oxidation products found in black tea, thearubigins, and theaflavins, also possessed antioxidant activity and retarded the development of lung cancer caused by NNK [45]. The administration of Polyphenon E and Caffeine not only reduced the incidence and multiplicity of lung adenocarcinoma in female A/J mice induced by NNK, but also inhibited cell proliferation, enhanced apoptosis, and lowered levels of c-Jun and ERK1/2 phosphorylation in adenocarcinomas and adenomas, suggesting that tea polyphenols (and perhaps caffeine) inhibit NNK-induced lung tumorigenesis [46].

*4.3. Vitamin.* Vitamin E inhibits tumor cell growth in vitro irrespective of its antioxidative effect. In NNK-induced lung tumors of mice, Vitamin E supplement reduced the mutation frequency of K-ras at codon 12, suggesting that it suppresses NNK-induced DNA injury [47]. $\alpha$-tocopheryloxybutyric acid (TSE), a nonantioxidative vitamin E derivative, could inhibit cell proliferation during the mouse lung tumorigenic process treated with NNK. The administration of Vitamin E or TSE suppressed the labeling index of the PCNA, the elevation of ornithine decarboxylase activity at a promotion phase of NNK-induced lung tumorigenesis [48, 49]. $\gamma$-tocopherol-rich mixture of tocopherols ($\gamma$-TmT, considered as vitamin E) significantly lowered tumor multiplicity, tumor volume, and tumor burden, which was associated with high apoptosis and low levels of 8-hydroxydeoxyguanine, $\gamma$-H2AX and nitrotyrosine in the NNK-induced lung [50].

Mice receiving the supplementation of 1$\alpha$,25-dihydroxyvitamin D3 (1,25D) had significantly lower tumor incidence and tumor multiplicity, but experienced body weight loss, kidney calcium deposition, elevated kidney CYP24 expression, and decreased fasting plasma 1,25D levels [51]. Inhaled mid-dose isotretinoin (13-cis-retinoic acid) caused up-regulation of lung tissue nuclear RARs relative

to vehicle-exposed mice [52]. 9-cis-Retinoic acid (9cRA) binds both RARs and retinoid X receptors and has been shown to be a potential chemopreventive agent. The mice receiving 9cRA supplementation had significantly lower tumor multiplicity and showed a trend toward lower tumor incidence, as compared with the mice given NNK alone [53]. The mice exposed to the high isotretinoin (13-cis retinoic acid) dose or 5-hydroxy-4-(2-phenyl-(E) ethenyl)-2(5H)-furanone (KYN-54, a novel retinoidal butenolide compound) showed reductions of tumor multiplicity after NNK treatment [54].

*4.4. Plant and Vegetable.* The discovery of dietary-related compounds with potential to inhibit lung cancer may present promising and practical approaches to reduce the risk of lung cancer caused by smoking. The exposure to fermented brown rice and rice bran significantly reduced the multiplicity, and tumor size of NNK-induced lung tumor with the expression of CYP 2A5 mRNA and Ki-67 protein decreased [55]. Deguelin, a natural plant product, specifically inhibits the proliferation of premalignant and malignant bronchial epithelial cells by blocking Akt activation [56]. Feeding with powdered adlay seed exerts an anticancer effect, evidenced by the reduced number of surface lung tumors [57]. The administration of Changkil saponins suppressed the NNK-induced increase in the level of PCNA and the number of lung tumors [58]. The treatment of 7-hydroxy-3-methoxycadalene from Zelkova serrata significantly reduced the incidence of adenomas and adenocarcinoma in a concentration-dependent manner [59]. Cinnamaldehyde (CNMA) treatment significantly reduced the combined incidence of adenomas and carcinomas, tumor multiplicity in transgenic rasH2 male mice [10]. Kava is a traditional beverage in the South Pacific islands and could prevent NNK plus BaP-induced lung tumorigenesis in A/J mice by enhancing apoptosis, inhibiting proliferation and the activation of NF-kappaB in lung tumors [60]. Isothiocyanates are derived from cruciferous vegetables and their N-acetylcysteine and phenethyl conjugates inhibit the formation of lung adenoma and adenocarcinoma with a significant reduction in PCNA and an induction of apoptosis in A/J mice induced by NNK [61, 62]. In (A/J $\times$ TSG-p53 "knockout") F1 mice with either the p53+/− or p53+/+ genotype, phenethyl isothiocyanate (PEITC) pretreatment significantly decreased tumor incidence and multiplicity [63]. $\beta$-carotene increased lung tumor multiplicity, lung tumor size, blood cell cAMP, serum, and lung levels of retinoids and induced p-CREB and p-ERK1/2 in PAC induced by NNK [64]. Treatments with Satsuma mandarin juice (MJ), MJ2, and MJ5 reduced the incidence and multiplicity of NNK-induced lung tumors by decreasing PCNA-positive index in lung tumors [65].

*4.5. Enzyme Inhibitor.* CYP enzymes can catalyze the $\alpha$-hydroxylation of NNK for its activation in the oxidative metabolism pathway, such as CYP2A6. A trend was noted for 8-Methoxypsoralen (8-MOP), an inhibitor of CYP2A6, to reduce adenomas and adenocarcioma to a greater extent than hyperplasia in mouse lung treated by NNK [66–69]. NNK is reported to promote COX-2 activity in colon and gastric cancer cells and the development of NNK-induced adenocarcinomas in mice is reduced by inhibitors of cyclooxygenase [70]. Another report showed that such specific COX-2 inhibitors as acetylsalicylic acid or N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfona-mide significantly increased the apoptotic index and inhibited the expression of COX-2 in NNK-treated mice [71]. Farnesyltransferase inhibitors (FTIs) included manumycin, gliotoxin, dihydroepiandrosterone, perillyl alcohol, and FTI-276. FTI-276 reduced both the tumor multiplicity and the total tumor volume/burden per mouse. The apoptotic index in FTI-276-treated tumors showed an increase of 77% over control tumors [72].

*4.6. Fatty Acid.* The supplementation of fish oil with a low $\omega$-6 (n-6)/$\omega$-3 (n-3) polyunsaturated fatty acid ratio was able to significantly decrease lung tumor prevalence compared to groups receiving soybean oil and corn oil, which was associated with increased expression of cell cycle inhibitor p21Cip1 and lipoxygenase isoform 15-LOX in the lungs [73]. The treatment of NNK increased the level of prostaglandin E2 as well as PCNA and induced the activation of an ERK cascade (ERK, MEK, and Raf-1) in high linoleic acid oil- (LA-) fed mice. On the other hand, oleic acid oil (OA) feeding abolished the NNK-induced activation of the ERK cascade. In conjugation with these events, OA feeding reduced lung tumor incidence and tumor multiplicity in mice compared with LA feeding. These results suggest that OA suppresses lung tumorigenesis and that this suppression is correlated with the inhibition of PGE2 production and inactivation of the ERK cascade [74]. Myoinositol in AIN-93 diet also proved to reduce the development of lung tumors induced by NNK [75].

*4.7. Anticancer Reagents.* The anticancer chemicals targeting the cell signals and metabolism can be employed to prevent carcinogenesis. Gefitinib is an EGFR tyrosine kinase inhibitor (EGFR-TKI) and could significantly suppress the multiplicities of the NNK-induced tumors in a dose-dependent manner [76]. The administration of Rapamycin, an inhibitor of mTOR, decreased tumor size, proliferative rate, tumor multiplicity, and mTOR activity in NNK-treated mice [77]. Targretin is a retinoid specifically selective for retinoid X receptors and widely used as an anticancer reagent. In mice, it could decrease the multiplicity and size of NNK-induced tumors, demonstrating its preventive and therapeutic activity [78]. Histone deacetylase inhibitors, such as suberoylanilide hydroxamic acid (SAHA), showed a significant inhibition of lung tumor multiplicity in mice treated with NNK. However, a significant inhibition of the alpha-hydroxylation pathway of NNK was observed in lung microsomes, suggesting that SAHA may act to inhibit the activation pathways of NNK metabolism [79].

## 5. Future Perspectives

The contribution of NNK to the imbalance between cellular proliferation and apoptosis, and subsequent lung tumorigenesis has been consistently described and confirmed in

numerous animal models. According to animal experiments, several mechanisms of NNK-induced lung carcinoma have been proposed, including (i) the activation of oncogenes via mutation, (ii) interruption and/or silencing of genes encoding enzymes coupled with NNK, (iii) direct manipulation of enzymes (specifically from the CYP protein family) responsible for activation and initiation of NNK-mediated processes, and (iv) the disruption of the signal pathways. Some primary preventive approaches have not yet been established, including (i) rendering NNK inactivation by antioxidants (tea, vegetable, vitamin and metal compunds) and (ii) obstructing the function of NNK (anticancer chemotherapeutic agents). In the recent years, too many transgenic mice has been bred and applied in the establishment of lung cancer model, which can provide an efficient tool for the investigation of lung cancer without the influence of chemical carcinogen. If treated with NNK, it is of great help and use to clarify the molecular mechanism of NNK-induced lung carcinogenesis and find out the novel target to prevent NNK-associated lung cancer. Therefore, it is necessary to delineate the most potent biomarkers of NNK-induced lung tumorigenesis, and to develop efficient methods to fight against this kind of lung cancer using animal model in the future.

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*Review Article*

# Paternal Smoking and Risk of Childhood Acute Lymphoblastic Leukemia: Systematic Review and Meta-Analysis

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*Objective*. To investigate the association between paternal smoking and childhood acute lymphoblastic leukemia (ALL). *Method*. We identified 18 published epidemiologic studies that reported data on both paternal smoking and childhood ALL risk. We performed a meta-analysis and analyzed dose-response relationships on ALL risk for smoking during preconception, during pregnancy, after birth, and ever smoking. *Results*. The summary odds ratio (OR) of childhood ALL associated with paternal smoking was 1.11 (95% Confidence Interval (CI): 1.05–1.18, $I^2 = 18\%$) during any time period, 1.25 (95% CI: 1.08–1.46, $I^2 = 53\%$) preconception; 1.24 (95% CI: 1.07–1.43, $I^2 = 54\%$) during pregnancy, and 1.24 (95% CI: 0.96–1.60, $I^2 = 64\%$) after birth, with a dose-response relationship between childhood ALL and paternal smoking preconception or after birth. *Conclusion*. The evidence supports a positive association between childhood ALL and paternal ever smoking and at each exposure time period examined. Future epidemiologic studies should assess paternal smoking during well-defined exposure windows and should include biomarkers to assess smoking exposure and toxicological mechanisms.

## 1. Introduction

Leukemia is the most common cancer in children and adolescents, accounting for about 1 out of 3 cancers in children [1]. Each year, around 3,250 children are diagnosed with leukemia, of which about 2,400 are acute lymphoblastic leukemia (ALL) cases [2]. In the USA, survival rate for children with ALL has improved markedly since the early 1970s and is now approximately 80%, but incidence rates have not decreased and have, in fact, increased by 0.8% annually from 1975 to 2007 [3]. Worldwide, according to the World Health Organization (WHO), there were 33,142 deaths from leukemia among children under age 15 in 2004, and childhood (<15 years) leukemia caused 1,228,075 disability adjusted life years (DALYs) [4]. Identifying risk factors for childhood leukemia is an important step in the reduction of the overall burden of childhood diseases.

Though it has been studied intensively, the etiology of childhood leukemia is not well established. A two-hit model was proposed by Greaves in which prenatal chromosome alterations and postnatal genetic alterations are necessary for childhood leukemia development [5]. Genetic susceptibility and environmental factors play potential roles in this process [6]. Ionizing radiation has been significantly linked to childhood leukemia [7]; evidence for an association with benzene exposure or with parental smoking and alcohol consumption is less convincing.

Multiple studies on parental smoking and childhood leukemia have been conducted in the past two decades, probably because tobacco smoke is a well-documented and prevalent carcinogen. Despite ongoing global efforts to reduce tobacco use, one billion men and 250 million women currently smoke worldwide [8], causing 5 million deaths and 57 million DALYs from cancer and other diseases each year [9]. In the USA, 46 million people or 24% of all adults smoke [10], which caused nearly half a million deaths and 5 million years of potential life lost each year from 2000 to 2004 [11]. In China, though smoking is uncommon among women, almost two thirds of men smoke [12], causing one million deaths each year to smokers [13] and 56,000 deaths and 480,000 DALYs from lung cancer and ischemic heart disease to nonsmokers [14].

At least 250 chemicals in tobacco smoke are known to be toxic or carcinogenic, including volatile organic chemicals like benzene, formaldehyde, aromatic amines, polycyclic aromatic hydrocarbons (PAHs), and nitrosamines and radioactive compounds like Polonium-210 [15]. Benzene has been shown to affect the blood-forming system at low levels [16], and formaldehyde has been shown to increase leukemia risk among exposed adults [17]. Smoking is causatively linked with adult leukemia [18], and secondhand smoke (SHS) is qualitatively similar in its chemical constituents to mainstream smoke [15], indicating that SHS exposure has the potential to cause adverse effect on the hematopoietic system. Children aged 6 to 11 years were reported to have urinary concentrations of the tobacco-specific carcinogen nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) nearly four times those of adult nonsmokers [19], indicating that children are less able to avoid exposure to SHS than adults. Smoking has also been shown to affect sperm morphology, motility, and concentration and to increase oxidative damage to sperm DNA [20]. Together, these findings indicate that parental smoking is a potential risk factor for childhood leukemia that could induce DNA damage and mutation pre- and postnatally. However, epidemiological studies on this topic have reported inconsistent findings. Through 2009, 20 studies [21–40] investigated maternal smoking and childhood ALL, with three studies [27, 36, 39] reporting statistically positive associations, two [32, 40] reporting statistically negative associations, and the remainder reporting nonsignificant association. Among the 18 studies on paternal smoking, eight showed increased risks of childhood ALL for at least one index (exposure level or time period) of paternal smoking [23, 27, 33, 34, 36, 37, 41, 42].

Given the extent of the exposure, the known carcinogenicity of tobacco smoke, and the inconsistent findings for paternal smoking and childhood leukemia risk, a thorough examination of the causal association between paternal smoking and childhood ALL, a major type of leukemia in children, is necessary. A recent meta-analysis by Lee et al. [42] found a significantly positive but weak association between paternal smoking preconception (but not after birth) and risk of childhood leukemia and ALL [42]. This analysis was based on 11 studies published from 1990 to 2008, plus their own study, results of which were published concurrently with the meta-analysis. However, Lee's meta-analysis did not include all published studies and did not sufficiently describe study exclusion criteria. Also, they did not look at confounding adjustments or perform assessment of dose-response relationships. Further, a new study [33] was published after this meta-analysis had been accepted for publication. Here, we conduct an updated and more comprehensive meta-analysis of the association between paternal smoking and childhood ALL based on 18 published studies that reported risk estimates or that provided data to calculate risk estimates. We examined risks associated with paternal smoking preconception, during pregnancy and after birth and, for the first time, analyzed dose-response relationships of exposure in these time windows.

## 2. Methods and Analysis

*2.1. Selection of Studies.* Preliminary literature searches were conducted by searching for the topics "smok*" or "tobacco" or "cigarette" and "leukemia" and "child*" in the ISI Web of Knowledge and PubMed databases. After duplicates were identified and removed, the titles and abstracts of the remaining records were examined, and all reviews and original epidemiologic studies investigating risk factors of childhood leukemia were included for further examination on the availability of information on paternal smoking and leukemia. The bibliographies or citations of all relevant articles were also searched and cross-referenced. Original epidemiologic studies published in peer-reviewed scientific journals or edited books, with data available on both childhood ALL and paternal smoking, were included.

To be included in this meta-analysis, studies had to fulfill three criteria: (1) reported estimates of association (odds ratio, OR, or relative risk, RR) of paternal smoking, and ALL, (2) reported estimates of variance (e.g., 95% confidence intervals (CI)) or included data needed to calculate it, and (3) did not present data from the same group of subjects as another publication used in the meta-analysis (in which case, the article with the most appropriate exposure assessment or published most recently was selected).

### 2.2. Data Analysis

*2.2.1. Definition of Exposure Time Windows.* Based on data available in the studies included in the analysis, three exposure time windows of paternal smoking with potential relevance to the development of ALL were defined, that is, *preconception*, *during pregnancy* and *after birth*. Summary effects were estimated for each of these three exposure windows. To estimate the overall summary effect of paternal smoking in any time window, the risk estimate was selected from each study included in the following order of preference: paternal ever smoking in lifetime, paternal smoking before conception, during pregnancy, and after birth. For the four studies which used exposure time windows covering more than one of these windows, the same estimate of risk was used for all the narrower time windows falling into the wider time window. For example, if a study presented only the relative risk estimate for paternal smoking in the year or 12 months prior to birth, that value was used to estimate the summary effect of both paternal smoking before and during pregnancy. In one study by Brondum et al. [21], the time window of pregnancy was further divided into three trimesters, and data were available only for each trimester rather than the whole time window. Since data were not available to combine the effects for all the three trimesters, and the relative risk reported for each trimester was almost the same, data from the first trimester was selected to represent this time window.

*2.2.2. Selection of Outcomes and Exposure Indices.* Most studies provided data on ALL specifically. In one study which reported the risk of three immunological subtypes of ALL, common-ALL, pre-B-ALL, and T-ALL, but not the risk of ALL overall, and no data were available to estimate the overall

relative risk of ALL [43], the estimate for the most common subtype (common-ALL, which comprised 66% of the ALL cases in the study) was selected.

When both multiple and binary exposure categories were available, the category with the highest exposure was selected to estimate the summary effect. Although multiple exposure indices were used in published studies, the majority of studies used the exposure index of cigarettes per day, thus the exposure index chosen from each study for this analysis was in the following order of preference: cigarettes per day (CPD), pack year (PY), number of smoking years, and smoker/nonsmoker. When both continuous and categorical exposure indices were available, categorical indices were selected for the point estimate and the continuous measures were used for dose-response analysis. When both current and ever smoking status were available, the current smoking status was selected.

*2.2.3. Calculation of Summary Effects.* Both fixed-effect and random-effect models were used to calculate summary effects. The fixed-effect model uses the inverse variance weighting method [44], and the variance (95% CI) of the summary effect estimate was calculated using the method presented by Shore et al. [45] if the estimate on the confidence interval was wider than the one estimated by the fixed-effect model itself. The Shore correction incorporates between-study heterogeneity and is usually more conservative than the fixed-effect model in estimating the variance. The random-effect model allows for the incorporation of between-study heterogeneity (if it is present) into the summary variance estimate (95% CI) [46]. Results from random-effect models were used for interpretations when between-study heterogeneity was statistically significant, otherwise results from fixed-effect models with Shore-corrected 95% CIs were used when the CIs were wider than the uncorrected ones estimated by fixed-effect models.

*2.2.4. Subgroup Analysis.* Analysis was also conducted to estimate summary effects for different study subgroups, such as those with the highest index of exposure categories, with adjusted risk estimates, with well-defined exposure time windows or with population based controls, in order to investigate the sensitivity of estimated summary effects to factors defining the subgroups.

*2.2.5. Dose Response Analysis.* All studies with dose-response data were included for review and analysis. ORs for paternal smoking of ≥20 CPD during each time window from different studies were extracted, or estimated if raw data were available, and combined to get a summary estimate of OR for paternal smoking of ≥20 CPD in this time window. Similarly, summary estimates of OR for paternal smoking of 10–19 CPD or <10 CPD (<20 CPD for the time window of after child birth) were calculated, and these summary ORs were plotted and compared for each time window.

*2.2.6. Heterogeneity Analysis.* Heterogeneity among studies was assessed using the general variance-based method as described by Petitti [47] and using the $I^2$ [48], which describes the percentage of total variation across studies that is due to heterogeneity rather than chance, and which is calculated as $I^2 = 100\% \times (Q - df)/Q$, where $Q$ is Cochran's heterogeneity statistic and $df$ (degrees of freedom), with negative values of $I^2$ set to zero. The Cochran's heterogeneity statistic is known to have low power of detecting true heterogeneity when the number of studies is small, while $I^2$ does not inherently depend on the number of studies in the meta-analysis [48]. Low, moderate, or high degree of heterogeneity was suggested to be approximated by $I^2$ values of 25%, 50%, and 75%, respectively [48].

*2.2.7. Analysis of Publication Bias.* Publication bias arises when studies with statistically significant positive results for exposure to environmental pollutants are more likely to be published and cited [49]. In this meta-analysis, publication bias was assessed by using funnel plots and Egger's and Begg's tests [50, 51] and by estimating the proportion of papers which reported statistically nonsignificant risk assessments.

Funnel plots (plots of effect estimates against sample size) are usually skewed and asymmetrical in the presence of publication bias and other biases [52]; Egger's test [51] is a linear regression approach to measure funnel plot asymmetry. Begg's test [50] assesses the interdependence of variance and effect size using Kendall's rank correlation test. This bias indicator makes fewer assumptions than that of Egger's test, but it is not sensitive to as many types of bias as Egger's test. If the number of studies included for Egger's or Begg's tests is small, the power of detecting publication bias could be very low [50, 51].

All data analyses described above were conducted using StataIC11.

## 3. Results

*3.1. Description of Studies Included for This Updated Meta-Analysis.* Twenty-one original epidemiology studies that examined risk factors of childhood leukemia and reported data on both ALL and paternal smoking were found. Three studies did not report relative risk of ALL for any index of paternal smoking or raw data to calculate the risk [53–55], and they were excluded for this study. Therefore, a total of 18 studies were included in the final analysis, and these studies are summarized in Table 1. The studies were conducted in 8 different countries, and their results were all published in peer-reviewed journals from 1990 to 2009. All studies were case-control studies, probably because childhood leukemia is too rare to conduct a cohort study. The age of childhood leukemia patient inclusion varies as detailed in Table 1, with studies including cases through age 18 months ($n = 1$), through age 9 years ($n = 1$), through age 14 years ($n = 10$), through age 15 years ($n = 4$), through age 18 years ($n = 1$), or unspecified with mean age 6.1 years and standard deviation 3.6 years for cases and mean age 6.6 years and standard deviation 3.5 years for controls ($n = 1$). Controls were recruited from the general population in all but three studies which used hospital-based controls [29, 31, 42]. All

Table 1: Description of the 18 original research studies on paternal smoking and childhood ALL included in the meta-analysis.

| Study | Cases/controls | Age (years) | Case recruitment | Control selection | Overall* | Before pregnancy | During pregnancy | After birth |
|---|---|---|---|---|---|---|---|---|
| Lee et al. 2009, Korea [42] | 106/164 | 0–18 | Incident childhood leukemia cases diagnosed in three hospitals in Seoul between 2003 and 2005 | Other patients from the three hospitals where cases came from, matched for age and sex | ≥400 cigarettes/life time | number of PYs, ≤10 or >10 PYs | smoking at home during pregnancy | number of PYs, ≤10 or >10 PYs |
| Rudant et al. 2008, France [33] | 647/1681 | <15 | Cases were identified directly by investigators with support of the national cancer registry in France between 2003 and 2004 | Population based, with quota match for age and sex | | CPDs from the year prior to the child's birth to the interview | CPDs from the year prior to the child's birth to the interview | CPDs from the year prior to the child's birth to the interview |
| MacArthur et al. 2008, Canada [28] | 351/399 | 0–14 | Incidence case from 5 regions in Canada, diagnosed between 1990 and 1994 | From health insurance roll, matched for age, sex, and area for each case | ever smoker | CPDs before pregnancy | CPDs in the year prior to the child birth | |
| Menegaux et al. 2007, France [30] | 407/567 | <15 | Cases derived from the national registry in 14 regions between 1995 and 1998 | Population based, frequency match for age, sex, and area | | CPDs in the 3 months before pregnancy | | CPD from the child's birth to the diagnosis |
| Chang et al. 2006, USA [23] | 228/306 | ≤15 | hospital diagnosed cases between 1995 and 2002, North California Childhood Leukemia Study | Random selection from birth certificates, individual match for age, sex, and maternal race | ever smoker: ≥100 cigs before diagnosis | CPD in the 3 months before pregnancy | | |
| Menegaux et al. 2005, France [31] | 240/142 | <15 | newly diagnosed acute leukemia cases from 1995 to 1999 in four cities in France | Mostly from departments of orthopedic of the same hospital, matched for age range | | | | CPDs from the index birth to interview |
| Pang et al. 2003, England [32] | 1375/6987 | <15 | National wide population-based cancer cases diagnosed by regional oncology units between 1991–1994 in Scotland and 1992–1994 in England | Randomly selected from Family Health Serves Authorities lists, and matched for sex, date of birth, and geographical area of residence | | ever smoked before conception | | |
| Sorahan et al. 2001, England [37] | 139/132 | <15 | Children first diagnosed with leukemia in 3 areas in England in 1980–1983§ | From General Practitioners list, matched for sex and date of birth | | CPDs | | |
| Infante-Rivard et al. 2000, Canada [26] | 486/486 | 0–9 | Cases from tertiary care centers for childhood cancers, diagnosed in 1980–1993, Quebec | Population based from family allowance, matched for age, sex, and area | | | | CPDs between birth and date of diagnosis |

Table 1: Continued.

| Study | Cases/controls | Age (years) | Case recruitment | Control selection | Overall* | Before pregnancy | During pregnancy | After birth |
|---|---|---|---|---|---|---|---|---|
| Brondum et al. 1999, USA [21] | 1618/1986 | <15 | Newly diagnosed with leukemia via clinical trial registries from 1989 to 1993, CCG study | RDD, individually matched on age, race, area code and exchange | smoking amounts during lifetime | ever smoked one month before pregnancy | ever smoked during the three trimesters | ever smoked during nursing period |
| Schuz et al. 1999, German [43] | 686/2588 | <15 | From a national wide cancer registry (1992–1997) and from cases diagnosed (1980–1994) and lived in vicinity of nuclear installations | randomly selected from complete files of local offices of registration of residents, matched for area, sex, and similar date of birth (within one year) | | CPDs in the last 3 months before pregnancy | | |
| Sorahan et al. 1997b, England [38] | 573/573 | <16 | Children who died from leukemia in England, Wales, and Scotland between 1971 to 1976 | From birth registers of local authority areas where cases died, matched by sex and date of birth | current status, 6 levels from 0 to 40 CPD | | | |
| Sorahan et al. 1997a, England [36] | 367/367 | <16 | Children who died from leukemia in England, Wales, and Scotland between 1953 to 1955 | From birth registers of local authority areas where cases died, matched for sex and date of birth | current status, 4 levels from 0 to 20 CPD | | | |
| Ji et al. 1997, China [41] | 114/114 | <15 | Newly diagnosed childhood cancer cases from 1985 to 1991 in Shanghai | Population-based controls from household registry, matched for sex, and year of birth | | PYs before conception | | PYs after birth |
| Shu et al. 1996, USA, Canada [34] | 191/363 | ≤18 months | infants newly diagnosed matched for leukemia from 1983 to 1988 via clinical trial registries | RDD, individually matched for year of birth, telephone area code, and exchange number | | CPDs in the month prior to pregnancy | CPDs during pregnancy | |
| Sorahan et al. 1995, England [35] | 371/371 | <16 | Children who died from leukemia in England, Wales, and Scotland between 1977 to 1981[§] | From the birth register of the local authority area in which the case child died, matched for sex and date of birth | | | CPDs during prenatal period, categorized into 6 levels | |
| John et al. 1991, USA [27] | 47/184 | 0–14 | Incident cases aged 0–14 diagnosed in Denver, Colorado from 1976 to 1983 | RDD, matched on age, sex, and geographic area. | | CPDs during the 12 months prior to birth | CPDs during the 12 months prior to birth | |
| Magnani et al. 1990, Italy [29] | 142/307 | 6.1/6.6[#] | Pediatric hospital prevalent cases in Turin Italy, diagnosed between 1974 and 1984 | Randomly sampled from medical or surgical wards of the same hospitals, no matches | | CPDs up to child's birth | CPDs up to child's birth | |

RDD: random digit dialing; CPD: cigarettes per day; PY: package year; *: overall status means without specific exposure time period specified; [§]: There was a small degree of overlap between cases included by Sorahan et al. 2001 [37] and cases included by Sorahan et al. 1995 [35]; [#]: mean age of cases at diagnosis: 6.1 years, with standard deviation of 3.6 years and mean age of controls: 6.6 years, with standard deviation of 3.5 years.

studies except the one by Magnani et al. 1990 [29] matched controls and cases by age and most studies also matched by gender and area of residency. Exposure information on paternal smoking was obtained primarily by interviewing the mother (11 studies [23, 29–36, 38, 42]), while the remaining studies interviewed both parents, when possible.

Three studies reported childhood ALL risks in relation to parental use of tobacco [35, 36, 38] using data from the same large project called the Oxford Survey of Childhood Cancers (OSCC). This survey interviewed the parents of all children who died of cancer (including leukemia) before their sixteenth birthday in England, Wales, and Scotland during the period 1953 to 1984 and parents of population-based healthy control children, matched for sex and date of birth [35, 36, 38]. Because each of the three papers reported results from different and non-overlapping subsets of data, they were regarded as independent and were all included in the meta-analysis. There was, however, a small degree of overlap between cases included by Sorahan et al. 1995 [35] from the OSCC and cases included by Sorahan et al. 2001 [37]. The later publication included 139 *newly diagnosed* childhood ALL cases less than 15 years old in three areas in England from 1980 to 1983 [37], and the early paper included 371 children who *died* from ALL before their sixteenth birthday in England, Wales, and Scotland between 1977 to 1981 [35]. Thus, there is potential overlap between newly diagnosed ALL cases and those who died from ALL during 1980-1981. Given the high five-year survival rate of childhood ALL during that time period in England (about 50%) [56], such an overlap would be expected to be very small in this 2 year period, thus, both the Sorahan et al. studies [35, 37] were included in this analysis.

Of the 18 studies included in the analysis, 6 reported data on the risk of childhood ALL associated with paternal ever-smoking throughout the lifetime [21, 23, 28, 36, 38, 42]. The summary effects of paternal smoking preconception, during pregnancy and after the child birth could be estimated from 13 studies, 8 studies and 7 studies, respectively. Menegaux et al. 2005 [31] reported that paternal smoking was not associated with ALL either before or during pregnancy, but did not provide the actual data. However, they did report data on the association during the period from the child birth to the interview. Thus, the Menegaux study (2005) was included to calculate the summary effects of exposure after birth only.

### 3.2. Estimates of Summary Effects, Subgroup Analysis and Heterogeneity Analysis

*3.2.1. Overall and Lifetime Paternal Smoking.* Results of the meta-analysis are presented in Figure 1 and Table 2. Figure 1 graphs the ORs (random effects analysis) generated by each meta-analysis and the ORs and weights of the individual studies included therein. Table 2 details the summary relative effects of paternal smoking overall and during specific time windows, and for different subgroups within these exposure windows, using both fixed effect and random effect models. The degree of heterogeneity associated with each measure is also provided. The summary effect for paternal ever smoking at any time period was 1.11 (95% CI: 1.05–1.18, $I^2 = 18\%$) based upon all 18 studies, shown in Figure 1(a) and Table 2. When analysis was restricted to the data from six studies on overall lifetime paternal smoking status, only, not during specific exposure windows, the summary effect decreased from 1.11 to 1.07 (95% CI: 1.01–1.14, $I^2 = 0\%$).

*3.2.2. Preconception Paternal Smoking.* The summary OR for risk of ALL associated with preconception smoking was 1.25 (95% CI: 1.08–1.46, $I^2 = 53\%$) based on 13 studies (Figure 1(b) and Table 2). When only the highest exposure indices available in 10 studies were included, the summary effect increased to 1.38 (95% CI: 1.11–1.72, $I^2 = 45\%$, Table 2). Exclusion from the analysis of studies with the largest or smallest OR, those with the highest weight, or those with hospital-based controls, did not have a large impact on either the summary effect estimates or the heterogeneity. Both the summary effect and the heterogeneity between studies decreased (1.17, 95% CI: 1.02–1.35, $I^2 = 33\%$) after removing studies with exposure time windows that spanned more than those defined in our analysis (with wide defined exposure time windows hereafter, for example, the year prior to the child's birth to the time of interview [33], 12 months prior to the child birth [27], or up to child's birth [29]). Exclusion of Rudant et al. 2008 [33] alone, whose estimated effect (OR = 1.7; 95% CI: 1.3–2.1) was for the highest paternal smoking ≥20 cigarettes/day from the year prior to the child birth to the time of interview, had a similar summary effect on OR and heterogeneity as removing all 3 studies with wide defined exposure windows. Five studies reported the effect of paternal smoking specifically in the last one or three months before pregnancy [21, 23, 30, 34, 43], and their summary OR was 1.13 (95% CI: 0.98–1.29) with no evidence of heterogeneity between studies ($I^2 = 0\%$).

*3.2.3. Paternal Smoking during Pregnancy.* Children whose fathers smoked while they were *in utero* had a 24% higher relative risk of getting ALL than those whose father did not smoke (summary OR = 1.24, 95% CI: 1.07–1.43, $I^2 = 54\%$), shown in Figure 1(c) and Table 2. When only the highest exposure indices available in four studies were included, the summary effect was 1.28 (95% CI: 0.93–1.76, $I^2 = 65\%$). Inclusion of only studies with adjusted ORs also increased the summary effect (summary OR = 1.34, 95% CI: 1.07–1.68, $I^2 = 60\%$). In contrast, when only studies with well-defined exposure time windows (during pregnancy) were included, the summary effect decreased to 1.15 (95% CI: 1.06–1.23, $I^2 = 0\%$). Exclusion of Rudant et al. 2008 [33] alone, which estimated the effect (OR = 1.7; 95% CI: 1.3–2.1) for the highest paternal smoking of ≥20 CPD from the year prior to the child birth to the time of interview, had a similar effect on OR and heterogeneity as removing all 3 studies with widely defined exposure window. Removing extreme ORs, studies with the highest weight or with hospital-based controls had little effect on the summary estimates (Table 2).

Table 2: Results of meta-analysis of paternal smoking in different time periods and childhood acute lymphoblastic leukemia (ALL) risk.

| Paternal smoking[a] | Studies included | $N$ | Fixed-effect model[b] | | Random-effect model | | $I^{2c}$ |
|---|---|---|---|---|---|---|---|
| | | | OR (95% CIs) | $P$ | OR (95% CIs) | $P$ | (%) |
| Paternal ever smoking | [21, 23, 26–38, 41–43] | 18 | 1.11 (1.05, 1.16) | .000 | 1.11 (1.05, 1.18) | .000 | 18 |
| Overall lifetime ever smoking[d] | [21, 23, 28, 36, 38, 42] | 6 | 1.07 (1.01, 1.14) | .027 | — | — | 0 |
| Preconception | [21, 23, 27–30, 32–34, 37, 41–43] | 13 | 1.16 (1.03, 1.31) | .016 | 1.25 (1.08, 1.46) | .002 | 53 |
| With the highest exposure index | [27–30, 33, 34, 37, 41–43] | 10 | 1.37 (1.13, 1.66) | .001 | 1.38 (1.11, 1.72) | .004 | 45 |
| Removing the smallest and greatest ORs | [21, 23, 27, 28, 30, 32–34, 41–43] | 11 | 1.16 (1.03, 1.31) | .013 | 1.25 (1.08, 1.46) | .003 | 50 |
| Removing the two greatest ORs | [21, 23, 27–30, 32–34, 37, 41–43] | 11 | 1.15 (1.03, 1.28) | .013 | 1.20 (1.05, 1.36) | .003 | 41 |
| Removing the highest weight[e] | [21, 23, 27–30, 33, 34, 37, 41–43] | 12 | 1.25 (1.08, 1.45) | .003 | 1.31 (1.10, 1.56) | .003 | 49 |
| Removing the OR from Rudant, 2008[f] | [21, 23, 27–30, 32–34, 37, 41–43] | 12 | 1.10 (1.00, 1.22) | .060 | 1.15 (1.01, 1.31) | .03 | 26 |
| With well-defined exposure period[g] | [21, 23, 28, 30, 32, 34, 37, 41–43] | 10 | 1.11 (0.99, 1.23) | .069 | 1.17 (1.02, 1.35) | .026 | 33 |
| With paternal smoking during 1 or 3 months before pregnancy | [21, 23, 30, 34, 43] | 5 | 1.13 (0.98, 1.29) | .085 | — | — | 0 |
| With population-based controls | [21, 23, 27, 28, 30, 32–34, 41, 43] | 10 | 1.16 (1.02, 1.31) | .020 | 1.25 (1.07, 1.45) | .005 | 54 |
| During pregnancy | [21, 27–29, 33–35, 42] | 8 | 1.19 (1.07,1.32) | .001 | 1.24 (1.07,1.43) | .004 | 54 |
| With the highest exposure index | [27–29, 33] | 4 | 1.34 (1.02, 1.77) | .037 | 1.28 (0.93, 1.76) | .13 | 65 |
| With adjusted ORs | [21, 27, 28, 33, 34, 42] | 6 | 1.26 (1.04, 1.51) | .017 | 1.34 (1.07, 1.68) | .010 | 60 |
| Removing the smallest and greatest ORs | [21, 27, 28, 33–35] | 6 | 1.19 (1.06, 1.33) | .002 | 1.25 (1.07, 1.45) | .003 | 58 |
| Removing the highest weight[h] | [21, 27–29, 33, 34, 42] | 7 | 1.23 (1.03,1.47) | .022 | 1.28 (1.04,1.58) | .022 | 59 |
| Removing the OR from Rudant, 2008 | [21, 27–29, 34, 35, 42] | 7 | 1.15 (1.06, 1.23) | .000 | — | — | 0 |
| With well-defined exposure period[i] | [21, 34, 35, 42] | 4 | 1.15 (1.07, 1.25) | .002 | 1.16 (1.03, 1.31) | .015 | 25 |
| With population-based controls | [21, 27, 28, 33–35] | 6 | 1.19 (1.06, 1.33) | .002 | 1.25 (1.07, 1.45) | .003 | 58 |
| After birth | [21, 26, 30, 31, 33, 41, 42] | 7 | 1.20 (0.97, 1.49) | .092 | 1.24 (0.96, 1.60) | .092 | 64 |
| With the highest exposure index | [26, 30, 31, 33, 41, 42] | 6 | 1.35 (1.06, 1.72) | .008 | 1.33 (1.00, 1.78) | .05 | 57 |
| Removing the smallest and greatest ORs | [26, 30, 31, 33, 41] | 5 | 1.32 (1.03, 1.70) | .027 | 1.27 (0.95, 1.68) | .10 | 58 |
| Removing the highest weight[j] | [26, 30, 31, 33, 41, 42] | 6 | 1.35 (1.06, 1.72) | .008 | 1.33 (1.00, 1.78) | .05 | 57 |

Table 2: Continued.

| Paternal smoking[a] | Studies included | $N$ | Fixed-effect model[b] OR (95% CIs) | $P$ | Random-effect model OR (95% CIs) | $P$ | $I^{2c}$ (%) |
|---|---|---|---|---|---|---|---|
| Removing the OR from Rudant, 2008 | [21, 26, 30, 31, 41, 42] | 5 | 1.05 (0.89, 1.23) | .58 | 1.06 (0.89, 1.26) | .25 | 12 |
| With population-based controls | [21, 30, 31, 33, 41] | 5 | 1.23 (0.96, 1.59) | .11 | 1.25 (0.92, 1.69) | .15 | 68 |

[a]When multiple indices of exposure categories were available, the highest was selected to estimate the summary effects; otherwise, the binary category was selected, except for the analysis of the subgroup with highest exposure;
[b]95% confidence interval (CI) and $P$ values were estimated by Shore correction when they were wider or greater than the unadjusted estimates by fixed-effect models. The Shore correction incorporates interstudy heterogeneity;
[c]$I^2 = 100\% \times (Q - df)/Q$, where Q is Cochran's heterogeneity statistic and df the degrees of freedom, with negative values of $I^2$ put equal to zero. $I^2$ describes the percentage of total variation across studies that is due to heterogeneity rather than chance
[d]Only included studies that reported an association between childhood ALL and paternal overall smoking status during lifetime
[e]Removed the OR from Pang et al. 2003, which accounted for 42% of the weight;
[f]Rudant et al. 2008 reported risk of childhood ALL for paternal smoking one year before the child birth to the time of interview; the same estimated risk was used for calculating the summary effect of childhood ALL for paternal smoking before conception, during pregnancy and after birth.
[g]Only included ORs for paternal smoking before pregnancy, removed the ORs from Rudant et al. 2008, John et al. 1991 and Magnani et al. 1990, which estimated ORs for paternal smoking in the year (12 months) before birth, preconception, and during the prenatal period;
[h]Removed the OR from Sorahan et al. 1995, which accounted for 70% of the weight;
[i]Only included ORs for paternal smoking during pregnancy and excluded ORs for paternal smoking during the year (or 12 months) prior to birth.
[j]Removed the OR from Brondum et al. 1999, which accounted for 35% of the weight.

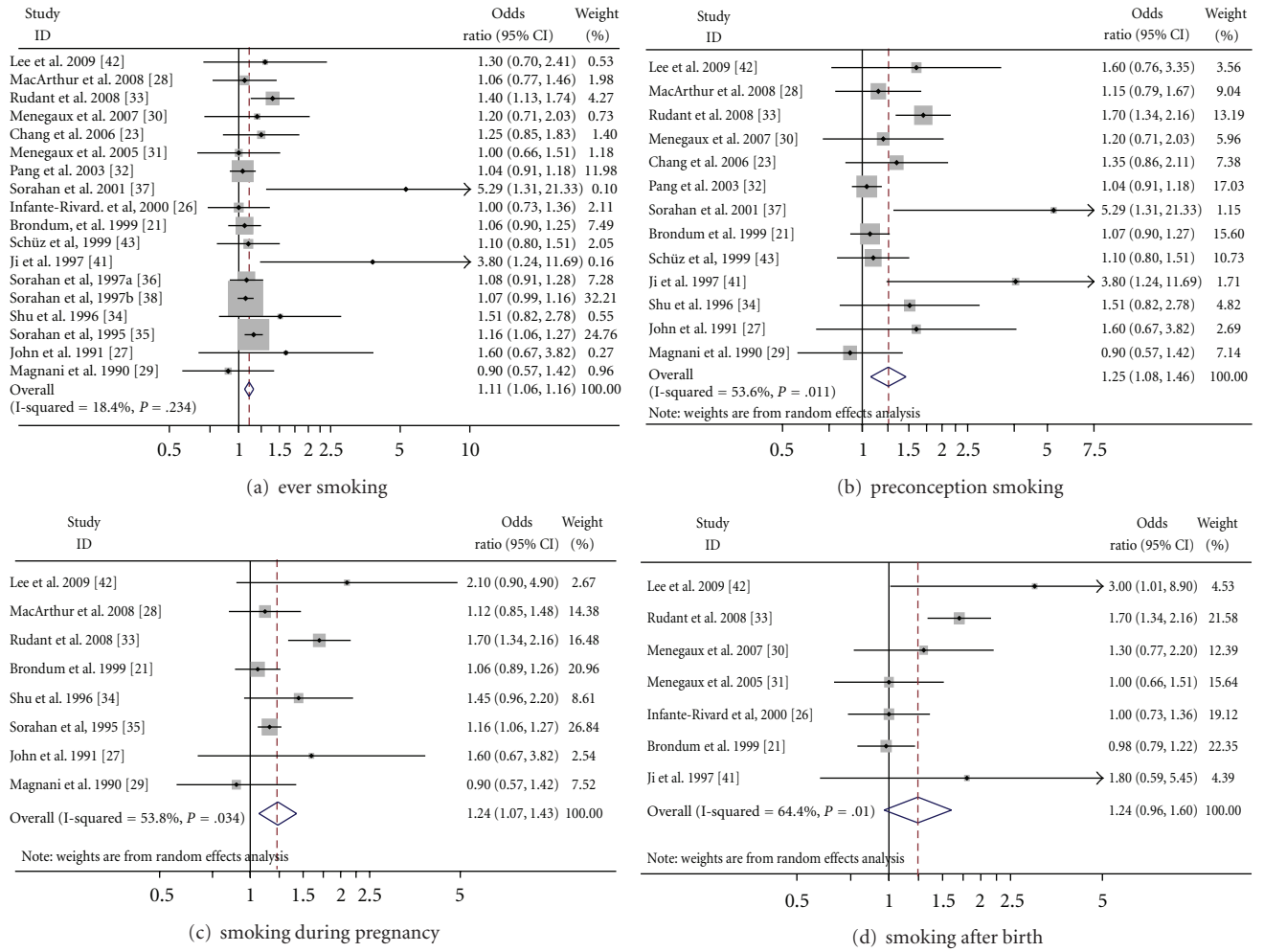


Figure 1: Meta-analysis of the association between childhood ALL and paternal smoking in different time windows. Random-effect OR estimates and weights were used in the graphs. $X$-axis represent the OR (odds ratio). The sizes of the boxes indicate the weight of the corresponding study used for estimates of summary effects.

Table 3: Summary of the five studies distinguishing the effects of paternal and maternal smoking on childhood ALL.

| Study | Exposure to parental smoking | | Number of case/control | OR (95% CI) | Adjustments |
|---|---|---|---|---|---|
| | Paternal smoking | Maternal smoking | | | |
| Lee et al. 2009, Korea [42]* | Ever smoked cigarettes | | | | Adjusted for age, sex, birth weight, father's education |
| | Lifetime nonsmokers | Lifetime nonsmokers | 22/41 | reference | |
| | ever | lifetime nonsmokers | 84/122 | 1.3 (0.7, 2.4) | |
| | pack-years before pregnancy | | | | |
| | 0 | lifetime nonsmokers | 22/41 | reference | |
| | ≤10 | lifetime nonsmokers | 48/60 | 1.6 (0.8, 3.1) | |
| | >10 | lifetime nonsmokers | 28/33 | 1.6 (0.8, 3.5) | |
| | Smoking at home during pregnancy | | | | |
| | Lifetime nonsmokers | lifetime nonsmokers | 22/41 | reference | |
| | yes | lifetime nonsmokers | 22/22 | 2.1 (0.9, 4.9) | |
| | pack-years after birth | | | | |
| | 0 | lifetime nonsmokers | 27/55 | reference | |
| | ≤10 | lifetime nonsmokers | 64/77 | 1.7 (0.9, 3.1) | |
| | **>10** | **lifetime nonsmokers** | **11/11** | **3.0 (1.0, 8.8)** | |
| Ji et al. 1997, China [41] | pack-years before pregnancy | | | | Adjusted for birth weight, income, paternal age, education, and alcohol drinking |
| | 0 | lifetime nonsmokers | — | reference | |
| | ≤2 | lifetime nonsmokers | — | 0.8 (0.2–2.5) | |
| | 2 to 5 | lifetime nonsmokers | — | 1.0 (0.4–2.7) | |
| | **≥5** | **Lifetime nonsmokers** | — | **3.8 (1.3–12.3)** | |
| | Pack-years after pregnancy | | | | |
| | 0 | lifetime nonsmokers | — | reference | |
| | ≤2 | lifetime nonsmokers | — | 1.1 (0.4, 2.8) | |
| | 2 to 5 | lifetime nonsmokers | — | 1.8 (0.6, 5.2) | |
| | ≥5 | lifetime nonsmokers | — | 1.8 (0.6, 5.5) | |
| Chang et al. 2006, USA [23] | No preconception smoking | no postnatal smoking | 144/205 | reference | Adjusted for household income and maternal smoking during preconception and pregnancy |
| | No preconception smoking | postnatal smoking | 8/27 | 0.72 (0.22, 2.38) | |
| | Preconception smoking | no postnatal smoking | 36/47 | 0.88 (0.51, 1.52) | |
| | **Preconception smoking** | **postnatal smoking** | **37/23** | **3.94 (1.25, 12.37)** | |
| Brondum et al. 1999, USA [21] | Never smoked in the home | never smoking in the home | — | reference | Adjusted for household income, mother's and father's race and education |
| | Never smoked in the home | ever smoked in the home | — | 1.10 (0.88, 1.38) | |
| | Ever smoked in the home | never smoking in the home | — | 1.04 (0.86, 1.26) | |
| | Ever smoked in the home | ever smoked in the home | — | 1.09 (0.91, 1.30) | |
| John et al. 1991, USA [27] | Not during the year prior to birth | not during the 1st trimester | — | reference | Adjusted for father's education |
| | Not during the year prior to birth | yes, during the 1st trimester | — | 1.9 (0.9, 4.1) | |
| | Yes, during the year prior to birth | not during the 1st trimester | — | 1.4 (0.6, 3.1) | |
| | Yes, during the year prior to birth | yes, during the 1st trimester | — | 1.8 (0.8, 4.0) | |

* It was reported that small portion of mothers smoked (the smoking rate was 6.1% for controls' mothers; it was not reported for cases' mothers).

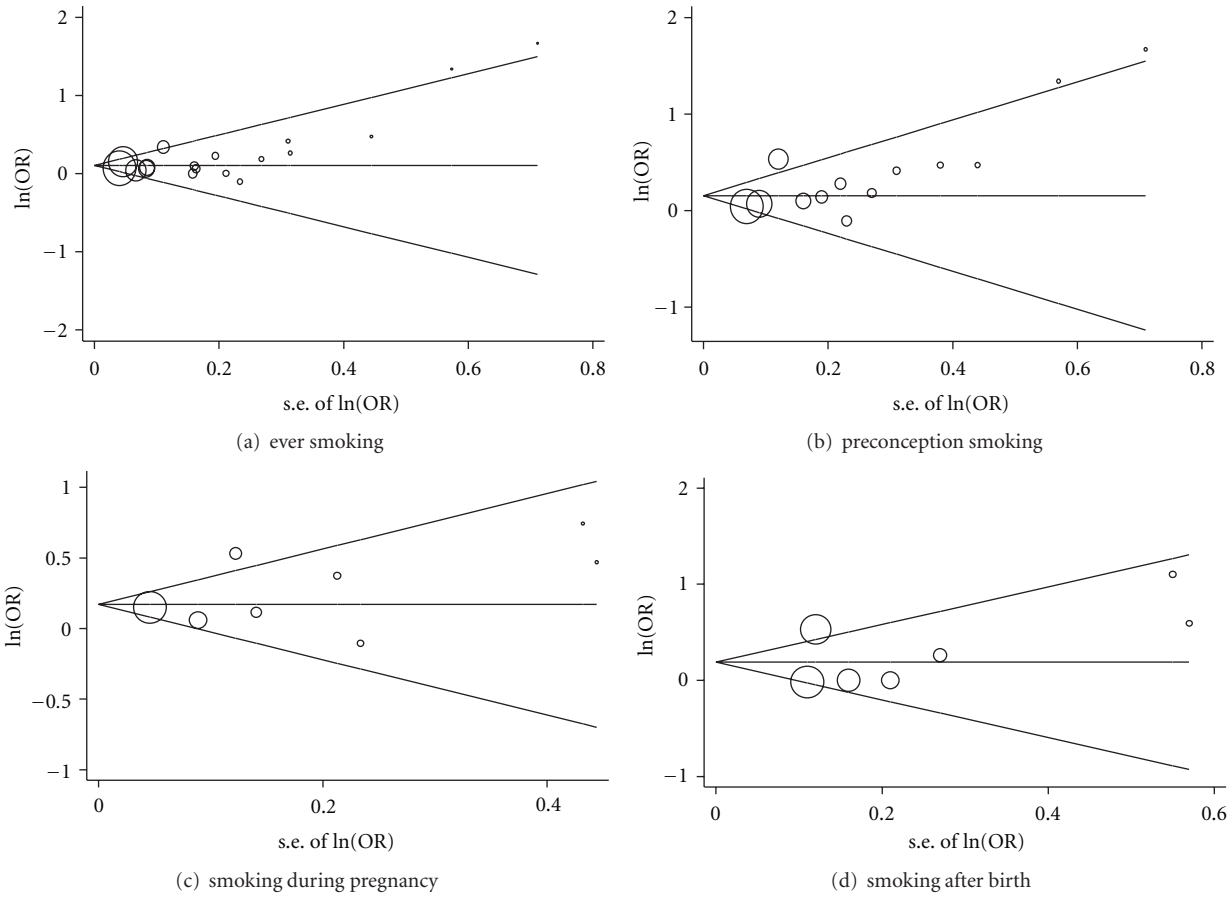


Figure 2: Begg's funnel plots of the natural log odds ratio (ln(OR)) versus the standard error of the log odds ratio (s.e of ln(OR)) of the studies used in the meta-analysis. Random-effect model OR estimates were used in the graphs. The sizes of the circles indicate the inverse-variance weight of the corresponding study.

*3.2.4. Paternal Smoking after Birth.* Paternal smoking after birth also had a positive but borderline significant association with childhood ALL (OR = 1.24; 95% CI: 0.96–1.60, $I^2$ = 64%), shown in Figure 1(d) and Table 2. Little difference was observed in the summary effects when removing extreme ORs or hospital-based studies (Table 2). When only studies with the highest exposure indices were included, or the study with the highest weight was excluded, the summary OR increased to 1.33 (95% CI: 1.00–1.78, $I^2$ = 57%). As with the other exposure windows, exclusion of Rudant et al. 2008 alone, reduced the OR, in this case, to 1.06 (95% CI: 0.89–1.26) and the heterogeneity to 12%.

*3.3. Effect of Adjustment for Confounding Factors.* One study [38] reported unadjusted RR, and two studies [35, 36] did not clarify whether they had adjusted for any other variables in their effect estimates. All remaining studies adjusted for at least some index of social economic status, for example, income or parental education, and most studies also adjusted for other potential confounders including residential area, birth weight, parental age and/or race/ethnicity, and alcohol drinking during pregnancy. For preconception paternal smoking, eight studies [23, 27, 28, 30, 33, 34, 37, 42] presented numbers of cases and controls with or without exposure allowing for the calculation of crude ORs. The current meta-analysis on these eight studies showed that the summary OR of the calculated crude ORs was 1.46 (95% CI: 1.18–1.80) and the summary OR of the reported ORs with adjustment was 1.37 (95% CI: 1.09–1.71).

*3.4. Analysis of Publication Bias.* For preconception paternal smoking, the $P$ values for Begg's and Egger's tests were 0.035 and 0.007, respectively, which, together with the funnel plots (Figure 2(b)), suggest some evidence of publication bias. This might be due to the inclusion of two relatively smaller studies with greater ORs and variance of estimates [37, 41]. Ji et al. 1997 [41] reported an OR of 3.8 (95% CI: 1.3–12.3) for children whose fathers smoked for 5 pack years before conception; Sorahan et al. 2001 [37] reported an OR of 5.29 (95% CI: 1.31–21.3) for paternal smoking with ≥40 CPD before pregnancy. Removal of these two studies resulted in a $P$ value of 0.24 for Begg's test and 0.07 for Egger's test, while the summary effect did not change much (random-effect model, summary OR = 1.20, 95% CI: 1.05–1.36, $P$ = .007, $I^2$ = 41%). Similar publication bias test results were found for paternal ever smoking in any time period, most likely

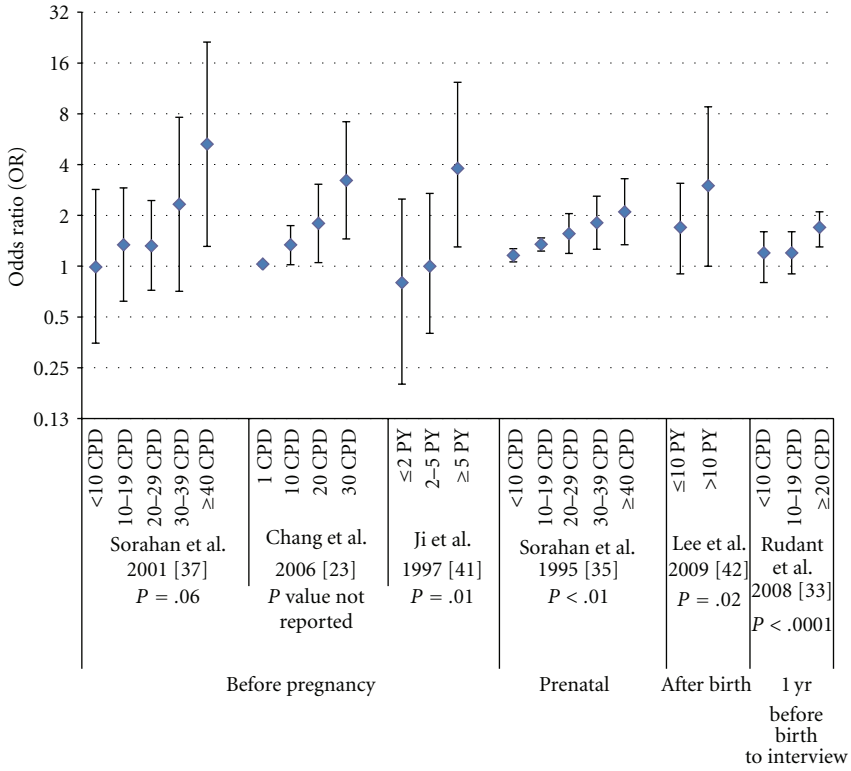


Figure 3: Clear positive dose-response associations between paternal smoking and childhood ALL found from the literature. Note: Among all the 18 studies included in this meta-analysis, two did not present dose-response analysis [32, 43], 10 did not find clear dose-response trend, and the remaining six studies reported clear dose-response trends [23, 33, 35, 37, 41, 42], which were presented in this figure. CPD: cigarettes per day; PY: pack years; the figure for Chang et al. 2006 [23] was estimated from their report that, for paternal preconception smoking, an OR of 1.03 (95% CI: 1.00–1.06) was associated with a one-CPD increment, and an OR of 1.34 (95% CI: 1.02–1.74) with 10-CPD increment, and the figure for Sorahan et al. 1995 [35] was estimated from their report of an OR of 1.16 (95% CI: 1.06–1.27) for change of one level of prenatal use of tobacco products.

because the same estimates from the Ji et al. 1997 [41] and Sorahan et al. 2001 [37] were included, Figure 2(a).

Both Begg's and Egger's tests showed no evidence of publication bias for studies on paternal smoking during pregnancy and after child birth (both with $P$ value $> .1$), though the power to detect publication bias might be lower because of the smaller number of studies included compared with preconception exposures, Figures 2(c) and 2(d). Nevertheless, the fact that only eight of the 18 studies included reported any statistically significant effect of paternal smoking on childhood ALL risk further indicates that the probability of publication bias is small.

*3.5. Dose Response Analysis.* Among the 18 studies included in this updated meta-analysis, two studies did not present dose-response analysis [32, 43] and 10 did not find significant dose-response relationships between paternal smoking and childhood ALL. Data from the remaining six studies [23, 33, 35, 37, 41, 42] that previously reported positive dose-response relationships are summarized in Figure 3. These data indicate that dose-response effects may occur before conception, during the prenatal period, or after birth.

We calculated the summary effects for exposure to different levels of paternal smoking during each of the three time windows, as shown in Figure 4. Data showed a positive dose-response relationship between childhood ALL and preconception paternal smoking, with a summary OR of 1.17 (95% CI: 0.9–1.54), 1.25 (95% CI: 1.01–1.55) and 1.30 (95% CI: 1.09–1.55), for paternal smoking of <10, 10–19, and ≥20 CPD, respectively. For paternal smoking during pregnancy, no dose-response relationship was found. For paternal smoking ≥20 CPD after birth, the summary effect was 1.24 (95% CI: 0.91–1.68), compared to the summary effect of 1.12 (95% CI: 0.83–1.51) for smoking <20 CPD.

## 4. Discussion

*4.1. Association of Paternal Smoking and Childhood ALL.* More than half of the studies included in this analysis reported relative risk estimates that were not statistically significant; one possible reason for this may be that a true association exists, but these studies did not have the sample sizes or statistical power to identify statistically significant associations. This is not surprising given the relatively low summary ORs we identified (i.e., <1.4) and the large sample sizes required to identify ORs of this magnitude in individual studies. Meta-analysis can increase study power by pooling all published data. The literature review and meta-analysis

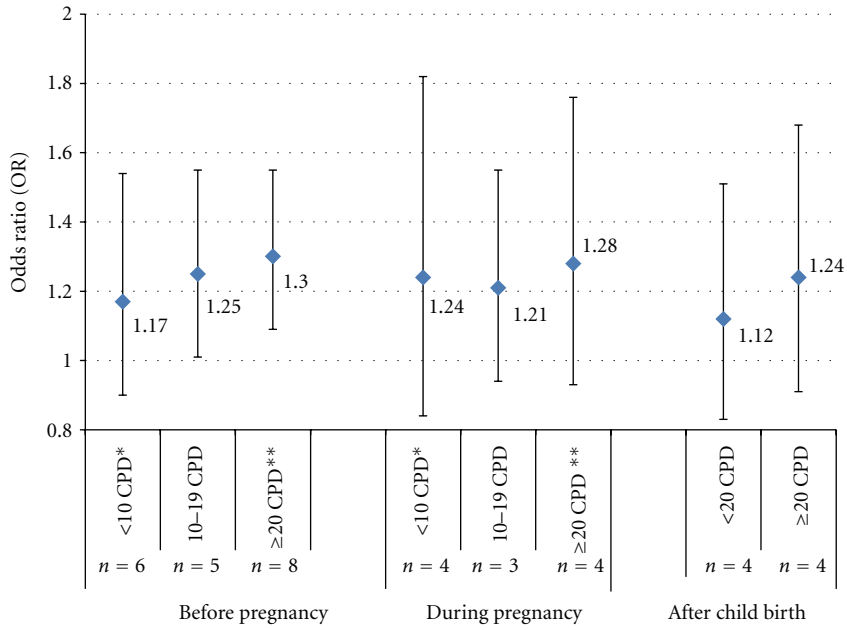


Figure 4: Evaluation of dose-response relationships between paternal smoking at different time windows and childhood ALL risk. Estimates by random-effect models were used when between-study heterogeneity was statistically significant; CPD: cigarettes per day; before pregnancy dose-response analysis was based on eight studies [27–30, 33, 34, 37, 43], during pregnancy analysis was based on four studies [27–29, 33] and after birth analysis was based on four studies [26, 30, 31, 33]; The risk estimates for exposure to paternal smoking with 10–19 CPD and with ≥20 CPD were combined to get estimates on exposure to paternal smoking with <20 CPD from Rudant et al. 2008 [33] and from Menegaux et al. 2005 [31]; *includes exposure to paternal smoking of 1–15 CPD (OR = 0.9, 95% CI: 0.6–1.5) up to child's birth, **includes exposure to paternal smoking of >16 CPD (OR = 0.9, 95% CI: 0.5–1.6) up to child's birth, as reported by Magnani et al. 1990 [29]; The dose-response analysis in Sorahan's paper in 2001 [37] was based on exposure categories of: lifelong nonsmokers, <10, 10–19, 20–29, 30–39, and ≥40 CPD during preconception, the later three categories were combined using the raw data for the purpose of this meta-analysis.

reported here, which incorporates more studies ($n = 18$) than previous reviews and finds positive dose-response relationships for exposure to paternal smoking before pregnancy and after child birth, supports statistically significant association between paternal smoking and childhood ALL. The International Agency for Research on Cancer (IARC) reviewed 6 studies published up until 2000 in 2004, and their meta-analysis for paternal smoking "*indicated no statistically significant association with acute lymphocytic leukaemia*" [18]. The Surgeon General Report 2006 [20] reviewed 10 epidemiology studies on parental smoking and childhood leukemia published from 1990 to 2001 and concluded that "*The evidence is suggestive but not sufficient to infer a causal relationship between prenatal and postnatal exposure to secondhand smoke and childhood leukemia.*" This report did not examine risk of ALL specifically. The California EPA also updated its review on parental smoking and childhood acute leukemia in its 2005 report [57], which included 13 studies and concluded that "*evidence to date is suggestive of an association between preconceptional paternal smoking and leukemia risk, but not postconceptional ETS (environmental tobacco smoke) exposure.*" These conclusions were all made on overall childhood leukemia including both ALL and AML and did not differentiate the potential effect of paternal smoking on childhood ALL specifically.

A recent meta-analysis by Lee et al. [42], based on 12 studies, found that the risk of childhood ALL increased with overall paternal smoking (OR = 1.07, 95% CI: 1.00–1.14, $n = 5$) and smoking before pregnancy (OR = 1.17, 95% CI: 1.04–1.30, $n = 9$) but not after birth. This research group did not evaluate risk during pregnancy. In contrast, in our current meta-analysis, overall lifetime paternal smoking and smoking preconception, during pregnancy and after birth, were all positively associated with childhood ALL. Further, positive dose-response relationships were found for exposure to paternal smoking before pregnancy and after child birth. Our findings, and those of Lee, strengthen the association of paternal smoking and childhood ALL overall, particularly preconception, while the effect of SHS during pregnancy and after birth on ALL risk requires further confirmation.

*4.2. Limited but Possible Confounding and Bias.* We assessed the strength of the observed associations in several ways. In general, moderate heterogeneity ($I^2 \approx 50\%$) was observed among studies. Selection bias was assessed by comparing summary effects estimated from all studies with those from studies including population-based controls only and was found to be minimal. Most studies analyzed matched cases and controls for gender and age and adjusted for SES and other potential confounders. Comparison of the summary effects of adjusted ORs with those of crude ORs in eight studies suggested that the adjustment of confounding does not impact the data but it is possible that some unmeasured or

residual confounders could have contributed to the observed effects.

Information bias is another potential issue as most of the studies collected data on paternal smoking from the child's mother, who might not be able to provide accurate information on the exposure. However, because the same approach was used for both cases and controls, information bias from this source was probably nondifferential and would likely bias the estimates towards null. Another source of information bias, recall bias, could arise from parents of cases being more likely to recall an exposure than parents of controls. However, smoking is not generally difficult to recall, so that recall bias is less likely than in studies of exposure to other environmental agents.

Both Egger's and Begg's tests indicated the probability of publication bias for paternal smoking before pregnancy, apparently due to inclusion of two studies [37, 41]. Ji et al. 1997 [41] studied the effect of paternal smoking for relatively longer periods or at higher exposures before conception (5 pack years before the conception); also, they obtained the exposure information by independent interviews with subjects' fathers and mothers so that the exposure assessment from this paper might be less biased than that from other studies. Sorahan et al. 2001 [37] examined the effect of paternal smoking with ≥40 CPD before conception, while the other studies generally used ≥20 CPD as their highest exposure group. Thus, the asymmetric funnel plot does not necessarily indicate evidence of publication bias, but may indicate possible heterogeneity or dose-response effects. The fact that many nonsignificant associations were published (56% of studies included in the current review) further indicates that the probability of publication bias is small or limited.

Maternal smoking is another potentially important confounding factor for the association between paternal smoking and childhood ALL. As data on maternal smoking was not adjusted in many of the studies included here, we were unable to estimate the summary effects of paternal smoking with complete adjustment for maternal smoking. However, the two studies in Asia [41, 42] were conducted in regions with very low smoking rates among women; in one study, none of the mothers of cases or controls smoked [41]; in the other study, only 6% of control mothers smoked [42], both reported positive associations (though not all were significant) between paternal smoking during some time windows and at higher levels of exposures and childhood ALL risk (Table 3). Three American studies [21, 23, 27], which examined both paternal and maternal smoking, found no evidence of an increased risk for exposure to only paternal smoking or only maternal smoking. But one study [23] found a significantly increased ALL risk for exposures to both parents' smoking (paternal preconception smoking and maternal smoking after birth). These five studies are detailed in Table 3. Since most of the studies on maternal smoking and childhood ALL found no association, it is unlikely that the results obtained in this meta-analysis were due to confounding effect by maternal smoking.

The potential effect of smoking in one time period on outcomes associated with another time period, is another confounding factor. Exposure to paternal ever smoking (in lifetime or during any of the three time periods) was positively associated with childhood ALL, but it was weaker than the effect of exposure in specific time windows, indicating a dilution of the effect of paternal smoking in the time window of interest by paternal ever smoking in lifetime. However, it was difficult to fully differentiate the effects of paternal smoking during different time periods in the current study. None of the studies looked at fathers who smoked exclusively during specific exposure windows or adjusted for paternal smoking in all other time periods. The study by Chang et al. 2006 [23] showed that, compared to children of lifetime nonsmoking fathers, the children of ever smoking fathers who did not smoke during the 3-month preconception period had an OR of 1.10 (95% CI: 0.63–1.91) while the children of fathers who smoked during the 3-month preconception period had an increased OR of 1.35 (95% CI: 0.86–2.10). Thus, including preconception paternal smoking in other time windows of interest might bias the risk estimate away from null.

*4.3. Potential Mechanisms of Action.* Different mechanisms likely underlie ALLs arising from exposure to paternal smoking pre- and postconception, and these are currently poorly understood. Active paternal smoking has been shown to deplete plasma and tissue antioxidant and increase oxidative damage to sperm DNA [58]. It has also been reported that mainstream tobacco smoke can cause paternal germ-line DNA mutation among mature male mice and that mutations accumulate in the spermatogonial stem cells with extended exposures [59]. Two published reviews found a suggestive causal relationship between paternal smoking and all childhood cancers, including also brain cancer and nerve system cancer, with significant increased risk of 10% to 20% [15, 60]. These lines of evidence provide biological plausibility that preconception paternal smoking can cause childhood leukemia. However, the elevated point estimate of the association between paternal smoking in the one or three months before conception was not statistically significant. This might be due to the low power of detection because of the small number of studies ($n = 5$) analyzed in this subgroup. Alternatively, this may indicate that the impact on sperm and short lifespan may not be restricted to exposure during this narrow preconception period. Cigarette smoking has been shown to alter gene expression patterns in airway epithelial cells, some irreversibly [61], and to alter microRNA expression profiling in bronchial cells, indicating possible epigenetic effects [62]. It is possible that sperm-producing cells are negatively impacted by persistent changes in gene or miRNA expression as a result of smoking at earlier times than three months before conception. Further studies are necessary to delineate the effects on sperm in well-defined windows of exposure before conception.

The biological mechanism underlying ALL arising from exposure during pregnancy or after birth could be mediated through changes in the lymphocyte transcriptome and subsequent effects on the immune system, as has been shown for active smoking [63]. A paternal effect *in utero* might

be expected to be weaker than a maternal effect. However, among the many studies which have investigated childhood ALL and maternal smoking during pregnancy, only three reported positive associations [27, 36, 39]. If carcinogenesis is not mediated by maternal smoking during pregnancy, it is less likely to be mediated by paternal smoking during this time window. A possible explanation for the positive association found in this meta-analysis is that most fathers who smoked during the pregnancy likely also smoked before the pregnancy or after birth. Paternal smoking status tends to be constant during different time periods [20]. This means that a risk apparently associated with smoking during or after pregnancy may have actually arisen from preconception exposure.

## 5. Conclusion/Impact

Evidence from the current meta-analysis strongly suggests a positive association between paternal smoking and childhood ALL. Given the high prevalence of smoking among males (35% in developed countries and 50% in developing countries [8]), the association with ALL is of great relevance to public health. Future molecular epidemiology studies should be designed with better assessment of paternal smoking during well-defined time windows. Given that smoking cessation is challenging, identifying the most relevant time window and motivating fathers to quit at least during that time window is one potential strategy to reduce the burden of childhood leukemia. Studies should also facilitate investigation of the underlying toxicological mechanisms, such as genotoxic, transcriptomic, or epigenomic effects on sperm or cord blood.

## Abbreviations

ALL: Acute lymphoblastic leukemia
CI: Confidence interval
CPD: Cigarettes per day
DALY: Disability adjusted life years
ETS: Environmental tobacco smoke
IARC: International Agency for Research on Cancer
OR: Odds ratio
OSCC: Oxford Survey of Childhood Cancers
PAHs: Polycyclic aromatic hydrocarbons
PY: Pack year
RR: Relative risk
SES: Social economic status
SHS: Secondhand smoke
WHO: World Health Organization.

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*Review Article*

# Epigenetic Effects and Molecular Mechanisms of Tumorigenesis Induced by Cigarette Smoke: An Overview

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Cigarette smoking is one of the major causes of carcinogenesis. Direct genotoxicity induced by cigarette smoke leads to initiation of carcinogenesis. Nongenotoxic (epigenetic) effects of cigarette smoke also act as modulators altering cellular functions. These two effects underlie the mechanisms of tumor promotion and progression. While there is no lack of general reviews on the genotoxic and carcinogenic potentials of cigarette smoke in lung carcinogenesis, updated review on the epigenetic effects and molecular mechanisms of cigarette smoke and carcinogenesis, not limited to lung, is lacking. We are presenting a comprehensive review of recent investigations on cigarette smoke, with special attentions to nicotine, NNK, and PAHs. The current understanding on their molecular mechanisms include (1) receptors, (2) cell cycle regulators, (3) signaling pathways, (4) apoptosis mediators, (5) angiogenic factors, and (6) invasive and metastasis mediators. This review highlighted the complexity biological responses to cigarette smoke components and their involvements in tumorigenesis.

## 1. Introduction

It is known that 90–95% of all cancers are caused by or closely associated with environmental factors and lifestyle. This includes diet (30–35%), cigarette smoking (25–30%), and alcohol consumption (4–6%) [1]. Cigarette smoking is an important risk factor for heart disease, chronic obstructive pulmonary disease, stroke, and acute respiratory diseases. In addition to all these noncancer diseases, it is also highly associated with human cancer development. The International Agency for Research on Cancer (IARC) identified cigarette smoking as the cause of cancer in more organ sites than any other human carcinogens. These include cancers of the lungs, oral cavity, larynx, nasal cavity, esophagus, stomach, pancreas, liver, kidney, urinary bladder, uterine cervix, and bone marrow [2]. There are over 5000 chemical compounds identified in tobacco and 62 of these have been evaluated by IARC as showing "sufficient evidence for carcinogenicity" in either animals or in humans [2, 3]. The major carcinogenic compounds include, but not limited to, radioactive polonium, N-nitrosamines such as 4-(methylnitrosaminao)-1-(3-pyridyl)-1-butanone (NNK), polycyclic aromatic hydrocarbons (PAHs) (e.g., benzo[a]pyrene (BaP)), and benzene [4]. A fine review on this aspect has been presented by Hecht in 2006 [5].

The carcinogenesis process is complex. Multistep processes of genetic and molecular defects have taken place before the manifestation of cancer [6]. Traditionally, there are three basic stages of carcinogenesis: initiation, promotion, and progression [7]. Carcinogenesis process is usually

accompanied by changes in structure and function of central genomic information coded in the DNA leading to various oncogene activations and tumor suppressor gene inactivations [8]. In addition, multiple signaling pathways may also be deregulated during the process of cancer development. Cancer growth also requires molecular changes that either affect the tumor cells themselves or alter the interaction between tumor cells and their surrounding stromal environment or the immune system. These events may eventually lead to tumor growth, invasion, and metastasis.

Cigarette smoke components have been reported to promote tumorigenesis by several mechanisms involving all three stages of carcinogenesis [5]. Genotoxic agents in cigarette smoke induce DNA damage through several mechanisms including gene point mutation, deletions, insertions, recombinations, rearrangements, and chromosomal aberrations. PAHs and nitrosamines are two of the most abundant genotoxic components in cigarette smoke. In addition to genotoxic effects, nongenotoxic effects of cigarette smoke are also extremely important. These effects can also act as modulators which alter cellular functions including cell proliferation and cell death. While synergistic effects of genotoxic carcinogens are known to occur, interaction between nongenotoxic (epigenetic) factors and genotoxic agents may also synergistically increase the risk for carcinogenesis [9]. The genotoxicity leading to carcinogenesis has been extensively reviewed in recent years [9–11]. In this present review, aside from a brief overview on the genotoxic effects of cigarette smoke components, we will provide a more extensive review on the non-genotoxic mechanisms of carcinogenesis by cigarette smoke or its components.

## 2. The Three Carcinogensis Steps Affected by Cigarette Smoke

*Step 1 (Initiation of Carcinogenesis).* Carcinogenesis may be the result of chemical or biological insults to normal cells through multistep processes that involves genomic changes (initiation of cancer development). Such changes eventually may also lead to cancer promotion and progression [12]. Some of the cigarette smoke components can act directly on DNA, but many require enzyme conversion before becoming carcinogenic [10, 11]. Most of such "conversions" involve metabolic changes via cytochrome *p*450s (*P*450s) such as *P*450s 1A2, 2A13, 2E1, and 3A4 to form the electrophilic entities that can bind to DNA to form DNA adducts. Such adduct formation is usually at the adenine or guanine sites of the DNA and lead to mutations such as those observed in the *KRAS* oncogene in lung cancer or those in the *TP53* gene in a variety of cigarette smoke-induced cancers [13, 14]. These mutation represent the so-called initiation step of carcinogenesis [15].

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN) are the most potent tobacco-specific nitrosamines in tobacco products and cigarette smoke. These compounds are formed from tobacco alkaloids like nicotine during the curing process of tobacco and are important tobacco carcinogens that can affect different tissues depending on the specific nitrosamines or their metabolites involved [5, 10]. NNK is a potent lung carcinogen but can also induce liver and nasal cancers. NNN has been shown to be carcinogenic to esophagus, nasal cavity, and respiratory tract in laboratory animals [16]. In humans, metabolites derived from NNK and the metabolites of NNK can also be identified in the smoker's urine [17].

Benzo[a]pyrene (BaP), one of the PAHs, is classified as a Group 1 carcinogen to humans [3]. It has been shown to have strong association and tumor-induction potentials in lungs, trachea, and mammary glands [5]. The carcinogenic potency of BaP has been demonstrated to be related to its metabolites which form DNA adducts with site-specific hotspot mutation in the *p53* tumor suppressor gene. Positive correlations of such adduct formation and tumor are indeed found in the lung cancer tissues of cigarette smokers [18].

These findings indicate that DNA mutations are increased in both tumor and nontumor bearing tissues of smokers. However, it must be pointed out that DNA adduct formations induced by cigarette smoke still cannot fully represent all the risk factors for cancer development in cigarette smokers [19]. For example, while there is higher incidence of pancreatic cancer in cigarette smokers than nonsmokers [20]. Assays for NNK metabolites in pancreatic cancer tissues in humans showed no significant difference between smokers and nonsmokers [21]. Thus, it is apparent that NNK-induced DNA adducts alone are not solely responsible for the pancreatic cancers in cigarette smokers. Nevertheless, NNK and its metabolite, NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol), are the only environmental carcinogens known to induce pancreatic cancer in animal models [22]. Thus, the contribution of NNK to pancreatic cancer in cigarette smokers still cannot be ignored. Furthermore, it is suggested that, in addition to DNA damage, synergistic interactions between DNA reactivity and epigenetic actions such as increased cell proliferation induced by NNK or by other chemicals in cigarette smoke may be needed for actual cancer development in such patients [23, 24]. There is indication that cigarette smoke carcinogens or cocarcinogen, such as nicotine, may also play a direct role to enhance cancer promotion and progression in human cancers after cancer development [25]. Such genotoxic mechanisms for cancer initiation and carcinogenesis by cigarette smoke components are well covered and discussed in several excellent reviews [5, 10, 11, 26–28]. Readers are encouraged referring to them. For the remaining portion of this article, we will provide more information on the non-genotoxic (epigenetic) mechanisms involved in cancer promotion and progression via cigarette smoke.

*Step 2 (Cancer Promotion).* Cancer promotion is characterized by deregulation of signaling pathways which control cell proliferation, apoptosis, and so forth, [29]. It is believed that although there are various genetic pathways which may lead to cancer development or cancer behaviors, there are certain hallmark capabilities or mechanisms which are commonly shared by all tumors. In the following discussion, we will describe each mechanism with illustrated examples.

*2.1. Effects of Cigarette Smoke on Self-Sufficiency in Growth Signals.* Normal cells need mitogenic growth signals to induce proliferation. These signals are transmitted into cells by receptors that bind distinct signaling molecules. In cancer cells, the receptors which transduce growth signals into cells are targets of deregulation during tumorigenesis. Receptor overexpression allows cancer cells to become hyperresponsive to low levels of growth factors that generally are not sufficient to trigger proliferation in normal cells [29]. Nicotine, a major component of cigarette smoke, is known to be a chemical that plays an important role in carcinogenesis in cigarette smokers [30]. Nicotine behaves like those growth factors which exert their biological functions mainly through the nicotinic acetylcholine receptors (nAChR) [31], $\beta$-adrenoceptors ($\beta$-AR) [32] or epidermal growth factor receptor (EGFR) [33]. The functions of these receptors are cell-type specific and the expression level and receptor sensitivity can be modified by nicotine. Obviously, alterations in either the receptor expressions or sensitivity play an important role in cigarette smoke-induced carcinogenesis [34–36].

Recent study by Lee et al. reported that $\alpha$9 nAChR expression in human breast tumors is elevated in advanced stages of breast cancer and plays important roles in human breast carcinogenesis [37]. Nicotine has been shown to mediate $\alpha$9 nAChR signaling and upregulate cyclin D3 expression in breast caner cells and breast cancer tissues [38]. Furthermore, it is also found that activation of the expression of $\alpha$9 nAChR by nicotine is through AKT signaling [39] and activation of $\alpha$9 nAChR signaling would elevate the phosphorylation status of adhesion molecule which plays a role in cancer metastasis [40]. Proliferation of mesothelioma cells is also found to be enhanced by nicotine [41]. This enhancement has been shown to be via $\alpha$7 nAChR with activation of ERK1/2 cascade as well as induction of NF-$\kappa$B and Bad phosphorylation. All these events eventually lead to inhibition of apoptosis [41] and increase of cancer risk. These findings were further supported by Wada et al. [42] who observed that nicotine promoted cell proliferation via $\alpha$7 nAChR mediated p44/p42-MAPK activation. Moreover, in our own study, we also reported that nicotine induced human bladder cells proliferation through ERK1/2 and Stat3 signaling downstream of $\alpha$7 nAChR and $\beta$-adrenoceptors ($\beta$-AR) [43]. In sum, all these studies indicate that nicotine, an important ingredient of cigarette smoke, promotes cellular proliferation which plays a critical role in carcinogenesis.

Other than nicotine, nitrosamines, such as NNK and NNN, also induced cancer cells growth through nAChR. NNK induced carcinogenesis by binding to nAChR especially for $\alpha$7 nAChR, whereas the biological impact of NNN is mainly modulated by $\alpha$4/$\beta$2 nAChR [8, 44–46]. It has been demonstrated that nicotine or NNK stimulated lung cancer cell proliferation via $\alpha$7 nAChR with activations of PKC, RAF1, AKT, ERK1/2, and transcription factors such as JUN, FOS, and MYC [47–49]. Question has been raised concerning the possibility that specific nAChR subunit upregulated by nicotine or NNK may be tissue specific or dependent. For instance, with nicotine or NNK, $\alpha$7 nAChR is the primary nAChR subunit which mediates tumorigenesis in lungs giving rise to pulmonary squamous cell carcinoma and mesothelioma [36]. On the other hand, $\alpha$9 nAChR is more associated with breast cancer [37]. Thus, the specific types of nAChR expressed in cancer cells may be considered as useful molecular targets for potential clinical therapy [50]. However, most of the nAChR present in cancer cells are still not functionally characterized yet. Future study will be needed to understand the functions of different nAChR subtype in cancer cells and the downstream signal pathways involved in tumorigenesis.

In addition to nAChR, a number of studies indicated that nicotine and NNK might also exert their biological activities through activation of receptors such as $\beta$-adrenoceptors ($\beta$-AR), EGFR, or insulin-like growth factor receptor (IGFR) or transactivation by nAChR signaling. It has been demonstrated that $\beta$-AR activation promotes the growths of various adenocarcinoma. For example, NNK can stimulate HT-29 cell proliferation through $\beta$-AR followed by cyclin AMP elevation and COX-2 expression [51]. Consistently, NNK stimulates the growth of pulmonary adenocarcinoma *in vitro* and *in vivo* via the release of arachidonic acid through COX-2 and 5-lipoxygenase (5-LOX) pathways that are mainly regulated by $\beta$-AR [52]. In another study by Schuller and Cekanova, NNK is reported to stimulate $\beta$2-AR receptor pathway (including PKA, cAMP, CREB) and transactivate EGFR pathway (such as Raf-1/ERK1/2 signaling) in the development of lung cancer [53]. It has also been reported that antagonists of $\beta$-AR can inhibit the development of NNK-induced lung adenocarcinoma [52]. Such antagonists are also found to be effective in reducing the stimulatory effects of nicotine on PKC, ERK1/2 activations, COX-2 expression, and gastric cancer cell proliferation [54]. Elevation of noradrenaline by nicotine via $\alpha$7 nAChR upregulation leading to significantly enhanced growth and angiogenesis in both gastric cancer and colon cancer has also been demonstrated [55]. Various investigators have also shown increases in neurotransmitters lead to $\beta$-AR activation, transactivation of EGFR, and the release of EGF [32, 54, 56]. Thus, an interrelationship between nAChR and neurotransmitter is apparent. Indeed, our recent investigation provided compelling evidence that chronic nicotine exposure induced release of noradrenaline via $\alpha$4/$\beta$2 nAChR activation followed by $\beta$-AR transactivation. Our study further demonstrated that blocking of $\beta$-AR with antagonist reversed the nicotine-induced cellular proliferative and chemoresistance [57].

Al-Wadei et al. first reported that nicotine contributes to the development of smoking-related pancreatic ductal adenocarcinoma (PDAC) with elevated levels of stress neurotransmitters (adrenaline and noradrenaline) and induction of cAMP, pCREB, and pERK1/2, and inhibition of $\gamma$-aminobutyric acid (GABA) [58]. GABA has been reported to possess tumor suppressor function suppressing both $\beta$-AR stimulated PDAC growth and migration *in vitro* [59]. However, while GABA is suppressed in PDACs, noradrenaline, PKA, p-CREB, and pERK1/2 in these tissues are overexpressed. A reduction of GABA by NNK is observed in lung adenocarcinoma [60]. These authors suggested that nicotine and NNK may contribute to the development of

PDAC in smokers by suppression of GABA with induction of stress neurotransmitters [61]. Schuller et al. further proposed that nicotine induces the release of stress neurotransmitters through activation of $\alpha 7$ nAChR and inhibits release of GABA via inhibition of $\alpha 4/\beta 2$ nAChR [61]. It is now believed that the stress neurotransmitter released via nAChR activation plays an important role in smoking-associated tumorigenesis [62]. However, the precise mechanisms involved in the regulation and the function of neurotransmitter released by nicotine and NNK are still uncertain. Future research on this area is encouraged.

It has also been shown that NNK can promote $\beta$-AR-mediated transactivation of EGFR followed by ERK1/2 phosphorylation leading to an increased proliferation in pancreatic cancer cells [63]. NNK is also reported to induce endogenous IGFR which is associated with the development of lung tumors [64]. Huang et al. also indicated that both activation of thromboxane A2 (TxA2) receptor and synthesis of TxA2 play critical roles in NNK-promoted lung cancer cell proliferation. TxA2 activates the transcriptional factor CREB through both ERK and PI3K/AKT pathways, which may also lead to PCNA and Bcl-2 overexpressions and cell proliferation [65]. These studies provide valuable information on the mechanisms which involve in proliferative signaling stimulated by nicotine and NNK through activation of nAChR, $\beta$-AR and other growth factor receptors in cancer cells. Triggering such receptors by cigarette smoke would further lead to rapid cell proliferation, cellular migration, invasion, and metastasis. In short, these investigations on the nAChR, and nAChR transactivated with other receptors represent the pivotal role in regulating multiple cellular cascades in general cell functions and in carcinogenesis.

Nicotine is also known to influence signal transducers and activators of transcription 3 (Stat 3) which is an important signal transducer mediating signaling by numerous cytokines, growth factors, and oncoproteins [66]. Findings from our own laboratories indicate that nicotine induces bladder cancer cells proliferation through $\alpha 7$ nAChR, $\alpha 4\beta 2$ nAChR, and $\beta$-AR followed by activation of ERK1/2 and Stat 3 [43]. Stat3 signaling further enhanced NF-$\kappa$B activation, cyclin D1 overexpression, and cell cycle progression [43]. Moreover, we also revealed that prolonged stimulation by nicotine upregulated $\alpha 4/\beta 2$ nAChR and $\beta$-AR followed with activation of Stat 3 leading to significant increase in chemoresistance in cells from bladder cancers [57].

In recent years, nongenotoxic actions of PAHs have gained increasing attentions. The biological effects of PAHs are mainly mediated via aryl hydrocarbone receptor (AhR). Through AhR, PAHs can then trigger ERK1/2 activation and signaling in hepatic "stem cell-like" epithelial cells [67, 68]. Other PAHs, such as benz(a)anthracene (BaA), has also been found to increase DNA synthesis and promote G1-S progression in serum deprived MCF-7 cells [69]. BaP has been shown to increase incidence of tumors in estrogen-responsive rodents, suggesting that it may also affect ER-mediated signaling [70]. PAHs can have actions which mimic those of estrogen. Some investigators believed that the estrogenic property of PAHs may be responsible for the induction of cell proliferation. BaP and BaA have been reported to act as estrogens that stimulate and initiate the ER-mediated transcription and cell cycle progression and enhance ER$\alpha$ phosphorylation [70]. On the other hand, there is also indication that the estradiol-dependent cell growth of MCF-7 cells can be inhibited by BaP and BaA [71, 72]. Thus, the actions of PAHs on estrogen-dependent cell proliferation are still controversial. Further studies are needed to elucidate more on the roles of PAHs in carcinogenicity.

*2.2. Effects of Cigarette Smoke on Antigrowth Signals.* In normal tissues, the antigrowth signals operate to maintain cellular quiescence and tissue homeostasis. Antigrowth signals can block proliferation by forcing the cell cycle progression into the quiescent (G0) state. The cell cycle transition from G1 to S phase is the key regulatory step in the cell cycle and is mainly regulated by CDK4/6-cyclin D and CDK2-cyclin E complexes. These complexes induce Rb phosphorylation and liberate E2Fs allowing cell proliferation to occur [73]. Disruption of the Rb pathway would therefore render cells insensitive to antigrowth factors [29]. Nicotine has been reported to induce binding of Raf-1 to Rb with activation of cyclins and CDKs as well as inactivation of Rb [74]. Via activations of nAChR and $\beta$-AR, nicotine and NNK both exhibit mitogenic properties by inducing cyclin D1 overexpression leading to G1/S transition and increasing cell cycle progression [49, 75, 76]. NNK can also stimulate normal human lung epithelial cells proliferation through NF-$\kappa$B and cyclin D1 upregulation in an ERK1/2-dependent pathway [75]. In our own laboratory, we have also demonstrated that nicotine-induced cyclin D1 overexpression is regulated via Stat3, ERK1/2, and NF-$\kappa$B-dependent pathways in bladder cancer cells [43].

Other study also shows that PI3K/AKT-dependent cellular proliferation is also enhanced in response to NNK [49]. The PI3K/AKT pathway is critical in cancer cells because it influences tumorigenesis, tumor growth, and therapeutic resistance [77]. The PI3K/AKT activation is documented in both NNK-treated A/J mice and in human lung cancers from smokers [48]. It also plays a role in NNK-induced cell transformation, proliferation, and metastasis [48]. It has been suggested that AKT and NF-$\kappa$B may serve as key targets for nicotine or NNK stimulation in the development of lung cancer [49]. West et al. also reported that BEAS2B cells treated with NNK for eight-week period increased cellular proliferation through activation of PI3K/AKT pathways [48]. However, PI3K/AKT activation does not always occur in all cancer cells induced by nicotine. Our previous study indicates that nicotine induced bladder cancer cell proliferation through Stat3 and ERK1/2 signalings instead of via AKT pathway [43]. All these investigations suggest that nicotine or NNK can activate ERK1/2, Stat3, or AKT signaling to interrupt the antigrowth signals leading to enhanced cell cycle progression and cancer promotion. It is important to remember that cigarette smoke components other than nicotine or NNK may also impede on antigrowth mechanisms enhancing cancer development and promotion. Such area of research also deserves focus in the future.

*2.3. Antiapoptotic Effects of Cigarette Smoke.* Apoptosis plays an important role in controlling normal development, homeostasis, and immune defense via elimination of redundant or abnormal cells in the organism [78]. Failure in cell elimination (reduction of apoptosis) may lead to undesirable cell survival and unchecked cell growths. Resistance to apoptosis is often seen in cancers where cancer cells tend to lose their proapoptotic potentials because of gene mutations. The most important gene mutations include tumor suppressor genes such as *p53*. Nicotine has been shown to inhibit apoptosis induced by tumor necrosis factor (TNF), by ultraviolet (UV), radiation, or by chemotherapeutic drugs such as cisplatin, vinblastine, paclitaxel, and doxorubicin [79]. This antiapoptotic action has been shown to be via PI3K/AKT, Raf/MEKK/ERK1/2, NF-$\kappa$B, Bcl-2, Bax, Bad, or surviving [23, 80–82]. West et al. demonstrated inhibition of apoptosis and promotion of proliferation in human bronchial epithelium cells by NNK are induced via activation of $\alpha 3/\alpha 4$ nAChR followed by upregulation of AKT, mitogen-activated protein kinase (MAPK), and PKC pathways [48]. Similar results are also observed by Xu and coworkers showing that both AKT and survivin pathways are involved in anticisplatin-induced apoptosis by nicotine [79]. Indeed, drug-induced enhancements of p53 and p21 expressions are shown to be suppressed by nicotine. This anti-apoptotic mechanism is mediated through $\alpha 3$ nAChR [83]. Our recent study also indicated that long-term nicotine treatment activated $\alpha 4/\beta 2$ nAChR and $\beta$-AR leading to reduction of apoptosis induced by cisplatin or paclitaxol [57]. Consistently, Zhao et al. also reported that nicotine induced up-regulation of Mcl-1 phosphorylation though ERK1/2 via $\beta$-AR activation with increased chemoresistance (anti-apoptosis) of human lung cancer cells [84]. Other investigators also indicate that NNK can prevent cell apoptosis by modulating the anti-apoptotic Bcl-2 and c-Myc proteins [23]. Heme oxygenase-1 (HO-1) is a protein induced during oxidative stress. It is found to be associated with cellular proliferation and is elevated during the developments of certain malignant tumors such as gastric and thyroid cancers [11–13]. Comparing the HO-1 in lung tissues of smokers and nonsmokers, Li et al. noticed that the expression of HO-1 is significantly increased in both tumor and nontumor tissues of smokers. These studies further revealed that NNK or its metabolites probably induce oxidative stress in lung tissues with elevation on stimulates the expression of HO-1. Such event is through ERK and NF-$\kappa$B activation and Bad phosphorylation induction leading to eventual apoptosis inhibition [11, 85].

Cell proliferation and apoptosis can also be modulated by the peroxisome proliferator-activated receptors (PPARs). PPARs are members of nuclear hormone receptor superfamily of ligand-dependent transcription factors. The major PPAR isoforms are $\alpha$, $\beta/\delta$, and $\gamma$ [86]. PPAR$\beta/\delta$ is expressed in most tissues and has been reported to be associated with cancer growths, especially those in liver, colon, breast and lungs [87–89]. Sun et al. reported a novel mechanism that nicotine increases PPAR$\beta/\delta$ expression through $\alpha 7$ nAChR follow by PI3K/mTOR activation leading to enhanced lung tumor cells proliferation [90]. In contrast to PPAR$\beta/\delta$, activation of PPAR$\gamma$ by its ligands induces apoptosis and inhibits cell proliferation [91]. Thus, an intact PPAR$\gamma$ levels or its activation is needed to reduce cancer risk (anti-apoptosis and cell proliferation). Interestingly enough, activation of PPAR$\gamma$ is found to be defective in lung cancers [92]. Furthermore, a significant reduction in the transcriptional activity of PPAR$\gamma$ and its endogenous ligands, including 15-S-Hydroxyeicosatertraenoic acid (15(S)-HETE) and 3-S-hydroxyocatadecadienoic acid (13(S)-HODE), are found reduced in lung tissues of NNK-treated mice. Indeed, lung tumors developed in these mice later. Yuan et al. further suggested that the reduction of 15(S)-HETE and 13(S)-HODE may enable lung cells to be more resistant to apoptosis by NNK and facilitate tumor development in the animals [93].

In contrast to nicotine or NNK, PAHs induce either apoptosis or antiapoptosis in mammalian cells [94, 95]. For instance, BaP is known to induce signaling through IGFR and increases cell survival through PI3K activation in human mammary epithelial cells [68]. Solhaug et al. reported that both AKT and ERK1/2 act as anti-apoptosis signals leading to Bad phosphorylation. However, BaP can also induce apoptosis through p53 and p21 signaling in the same model [96]. The results suggest that BaP is capable in stimulating both apoptosis and anti-apoptosis signals. Teranishi et al. reported that light-irradiated BaP (LBaP) inhibited apoptosis through production of ROS from degraded BaP [97]. This anti-apoptotic signal induced by BaP in combination with DNA damage would increase the possibility of cell survival and producing mutations. Thus, while the apoptotic signal of BaP induces cell death (cytotoxicity), the anti-apoptotic signals of BaP play an important role in cell proliferation and carcinogenesis. The precise factors influencing either apoptotic or anti-apoptotic outcome are still unclear. The anti-apoptosis mechanisms induced by components of cigarette smoke are obviously quite complex. It is evident that evading apoptosis plays a critical role in cigarette smoke-induced tumorigenesis and chemoresistance. Further explorations are very much needed. New understandings on the molecular target regulating the apoptotic and anti-apoptosis machineries by cigarette smoke could provide novel strategies for drug development with substantial therapeutic benefits.

*2.4. Effects of Cigarette Smoke on Replicative Lifespan.* When a cell population has progressed through a certain number of doublings (replications), they would normally stop growing and enter into a process called "senescence". Tumor cells, however, appeared to have limitless replicative potentials (immortalization) during tumor progression [29]. Telomeres, which define the end segments of chromosomes, consist of short, tandemly repeated DNA sequences $(TTAGGG)n$ together with associated proteins. They represent important devices in controlling cell divisions and proliferations. Small amount of these end DNA sequences may be lost during each cell cycle as a result of incomplete DNA replication. However, de novo additions of TTAGGG repeats by the enzyme telomerase may compensate for this loss [98]. Thus, telomerase plays an important role in the maintenance

of the telomere ends in normal cells. Ectopic expression of telomerase would immortalize the cells.

By using human tissue samples, Yim et al. reported that there are different distributions of the telomerase activity between smokers or ever-smokers and non-smoker. A strong correlation between telomerase activity and the number of packs years smoked can be established among these subjects indicating that there is an association between tobacco exposure and telomerase activity in the human bronchial epithelium. Increased telomerase activity would extend the "lifespan" of cells and put these cells to be at higher risks for malignant transformation and carcinogenesis [99]. Similar finding is reported by Targowski et al. that extensiveness of tobacco smoking correlated positively with increases in telomerase activity in tumor cells from patients with non small cell carcinoma of the lungs [100]. All these studies point to the fact that enhancement of the telomerase activity by cigarette smoke certainly underlies the cancer promotion potentials of cigarette smoke. However, which components in cigarette smoke altered telomerase activity are still not known. Further study in this aspect is very much needed.

*2.5. Effects of Cigarette Smoke on Mobilization of Cellular Resources.* Tumorigenesis requires adequate ability for protein synthesis and the energy for activating signaling. Indeed, there are indications that certain protein synthesis and mitochondria play central roles in neoplastic transformation. It is well known that mTOR and MAP kinase signaling pathways modulate the phosphorylation of transcriptional factors, stability of mRNAs, and protein synthesis [101]. Jin et al. reported that both nicotine and its metabolite NNK can induce survivin mRNA expression through AKT-mTOR and mediated *de novo* synthesis of survivin protein in normal lung epithelial cell HBE cells. This induced survivin expression has been claimed to play a role in the malignant transformation of HBE cells by stimulating the survival pathways [102].

Cigarette smoke may damage respiratory chain function in mitochondria enhancing oxidative stress leading to mitochondria dysfunction [103, 104]. It has also been reported that nicotine exposure resulted in reduced pancreatic mitochondrial enzyme activity, degranulation of beta cells, elevated islet oxidative stress, and impaired glucose stimulated insulin secretion in rats [105]. Continued exposure to ROS and free radicals from such "mitochondrial stress" may lead to mitochondria DNA (mtDNA) mutation which may play an important role in carcinogenesis [106]. Analyzing clinical samples, Tan et al. demonstrated mtDNA mutation in buccal cells of smokers [107]. Petros et al. also showed that tumor cells with mtDNA mutations grow faster then cells without mitochondrial mutation [108]. Hence, it is apparent that cigarette smoke would induce oxidative damage to the mtDNA leading to more aggressive tumor growths. Impact of cigarette smoke or its components on mitochondrial dysfunction needs further exploration.

*Step 3 (Cancer Progression).* The "malignancy" of a tumor is usually evaluated by its ability in invasion and metastasis as well as in the associated angiogenesis. There are ample evidence which indicate that cigarette smoke participates in the processes of angiogenesis, invasion, and tumor metastasis. These phenomena are presented and discussed below.

## 3. Effects of Cigarette Smoke on Sustained Angiogenesis

Angiogenesis, the development of new blood vessels from endothelial cells (ECs), is a critical event which allows the cancer cells to receive adequate nutrients and oxygen. Angiogenesis involves mature vascular changes, including detachment of pericytes, degradation of extracellular matrix, endothelial cells remodeling, proliferation, migration, and formation of new endothelial cells into tubular structures [109]. Survival and proliferation of vascular endothelial cells are often stimulated by tumor-derived mitogens, and vice versa. Tumor cells are known to activate angiogenesis by changing the balance of angiogenic inducers such as VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor), and by countervailing inhibitors such as thrombospondin-1 [29]. VEGF promotes angiogenesis and lymphangiogenesis in tumors, providing routes for dissemination. It has been shown that nicotine can induce angiogenesis both *in vitro* and *in vivo* and contributes to the growth of tumors [30, 110]. Similar to the FGF, nicotine is found to have the ability to promote migration, proliferation, tube formation and nitric oxide (NO) production of endothelial cells [111]. NO is a well-known vasodilator and angiogenesis mediator, and nicotine has been reported to enhance the expression of endothelial nitric oxide synthetase and promote NO release [110].

Nicotine is also found to induce expression of endothelial growth factors such as VEGF, bFGF, PDGF, TGF-$\alpha$, and TGF-$\beta$ in endothelial cells and smooth muscle cells [112, 113]. Enhanced bFGF release and increases in metalloproteinase expression with degradation of ECM have been demonstrated with nicotine [114, 115]. Moreover, nicotine is found to induce secretion of prostacyclin which is a vasodilating molecule associated with endothelial cell proliferation, survival and migration [116]. These effects are believed to be associated with cigarette smoke-induced hyperplasia of the intima in the blood vessels and other vascular wall lesions [115].

Tumor angiogenesis can also be modulated by the nAChR [117]. $\alpha 7$ nAChR is important in both physiological and pathological angiogenesis [110, 118]. $\alpha 7$ nAChR in endothelial cells needs to be sensitized or activated by hypoxia or ischemia in order to induce angiogenesis [110]. Indeed, specific antagonist of the $\alpha 7$ nAChR ($\alpha$-bungaratoxin) is shown to inhibit nicotine-induced angiogenesis (new vascular tube formation from endothelial cells) [25, 114]. Interestingly enough, it is apparent that the AKT pathway is found to be not involved in either angiogenesis or VEGF release induced by nicotine [25]. In contrast, Heeschen et al. suggested that inhibition of ERK1/2, p38 MAPK, and PI3K/AKT can completely block and prevent

endothelial tubule formation induced by nicotine-triggered $\alpha 7$ nAChR activation [110]. Consistent with Heeschen's study, Zhang and coworkers reported that nicotine apparently increases angiogenesis and invasion by activating PKC, PI3K/AKT, ERK1/2, mTOR, and Src in human NSCLC [119]. Excellent reviews on angiogenesis induced by nicotine were recently published [120, 121] and will not be further discussed here.

Interaction between nAChR and the growth factor-mediated angiogenesis occurs at signaling and transcription levels. Nicotine-induced expression of VEGF has been shown to be regulated by EGFR transactivation and via the ERK1/2 pathway in smooth muscle cells [122]. Phosphorylation of the VEGF receptor KDR by nicotine activates VEGF and increases its activity [112]. Additionally, nicotine can also upregulate the expression of VEGF receptor VEGFR2 during angiogenesis in certain cancer cells [123]. Recent study further indicated that nicotine can synergistically promote the proangiogenic effect of estradiol in nonsmall lung cancer [124]. Induction of angiogenesis in colon cancer by nicotine via $\beta$-AR followed by arachidonic acid pathway has also been reported [32, 125].

In sum, $\alpha 7$ nAChR subtype has been linked to angiogenic process induced by nicotine leading to tumor vascularity, inflammation, and ischemia. Nevertheless, whether nicotine or NNK acts specifically via nAChR or $\beta$-AR receptors or both or whether it is controlled in a cell-specific manner needs further study. Other components present in cigarette smoke that may also contribute to angiogenesis remain to be identified. The significant role of nAChR in various pathogenic angiogenesis is still largely unknown. This information would be critical for the development of new anti-angiogenic therapies. Several excellent reviews on the roles of nicotine and nAChR in angiogenesis exist [117, 120, 121, 126]. Readers are encouraged to refer to them for more detailed information.

## 4. Effects of Cigarette Smoke on Cancer Invasion and Metastasis

The ability of invasion and metastasis allows cancer cells to escape from the primary tumor mass to new terrains in the body. Metastasis is the final and most devastating consequence in malignancy. The processes of invasion and metastasis are exceeding complex. The genetic and biochemical determinants as well as the molecular mechanisms involved are still poorly understood. Many evidence indicate that cigarette smoking not only increases proliferation of cancer cells but also promotes metastasis [127]. Clinical and epidemiological studies suggest that smokers have more rapidly progressing tumors and cancer metastasis than nonsmokers [128]. These processes are now known to be dependent on cellular and stromal interactions and on extracellular matrix degradation. E-cadherin is a cell-to-cell interaction molecule expressed on epithelial cells. A loss of E-cadherin is seen in epithelial to mesenchymal transition (EMT), which is a major pathologic event in cancer metastasis. Chronic treatment of nicotine downregulated the expression of ECM proteins such as E-cadherin and $\beta$-catenin with concomitant increases of fibronectin and vimentin in lung cancer cells [129]. Wei et al. also indicated that NNK enhanced colon cancer cell migration with downregulation of E-cadherin. This author also found that the expressions of Snail and ZEB1, 2 major transcription repressors of E-cadherin, were also induced by NNK in colon cancer cell cultures [44]. Contactin-1 is a glycophosphotidylinositol (GP)-anchored adhesion molecule. Its upregulation is significantly linked with tumor progression, metastasis and poor prognosis in lung cancer patients [130]. It has been shown that NNK can upregulate contactin-1 via $\alpha 7$ nAChR/ERK activation and enhances invasiveness of lung cancer cells [131].

The second general mediators for invasion and metastasis are the extracellular proteases [29]. Breakdown of the extracellular matrix (ECM) through a family of enzyme called matrix metalloproteinases (MMPs) is needed for tumor cells to invade adjacent tissue and to metastasize. Zong et al. reported that nicotine enhanced the invasiveness of esophageal squamous carcinoma cells (TE-13) by up-regulating the expressions and activity of MMP-2, and COX-2 [132]. Nicotine is found to enhance the activity of MMP-2, and MMP-9 as well as activation of plasminogen activators in a COX-2 and VEGF-dependent manner [123]. Osteopontin (OPN) is a proinflammatory and pro-metastatic protein. It can be upregulated by nicotine. It serves as a good marker for PDAC (pancreatic ductal adenocarcinoma) metastasis especially in cigarette smoking population [133]. In a recent investigation, Lazar et al. demonstrated that nicotine contributes to PDAC metastasis through the induction of MMP-9 and VEGF mediated by OPN [134].

PAHs, including BaP, are also found to play a role in the promotion of cancer metastasis. Through augmented COX-2 expression and PGE2 production via activated AhR pathway, BaP induces breast cancer cell invasions [135]. BaP and PAHs mixture has also been demonstrated to induce cancer cell invasions and metastasis through upregulating the expressions of MMPs, proteinase-activated receptor-2, fibronectin, migration stimulating factor, and Bcl-2 protein in lung adenocarcinoma [136]. The importance of FGF-9 and its up-regulation by BaP in lung cancer invasion and metastasis has been proposed. Indeed, recent study by Ueng et al. [137] demonstrated that BaP increases the invasive potential of lung cancer cells *in vitro*. Such process involves the up-regulation of FGF-9 mRNA expression via the p38 and ERK1/2 pathways [137].

During metastasis, the cancer cells co-opt signals that control leukocyte trafficking and chemokines-mediated cell migration [138]. Among these chemokines, CXCR4 and its natural ligand CXCL12 serve as key mediators for tumor migration and metastasis [139]. Nicotine has been shown to increase the expressions of several CXC chemokines receptors such as CXCR2, CXCR3, and CXCR4 as well as CCL12 in SCLC cells [140] suggesting the nicotine would stimulate cancer cell migration and eventual metastasis.

Although epidemiology studies have long demonstrated the relationship between smoking and cancer metastasis, the molecular mechanisms of metastasis influenced by cigarette smoke or its components remain very limited. Further studies in this subject are urgently needed.

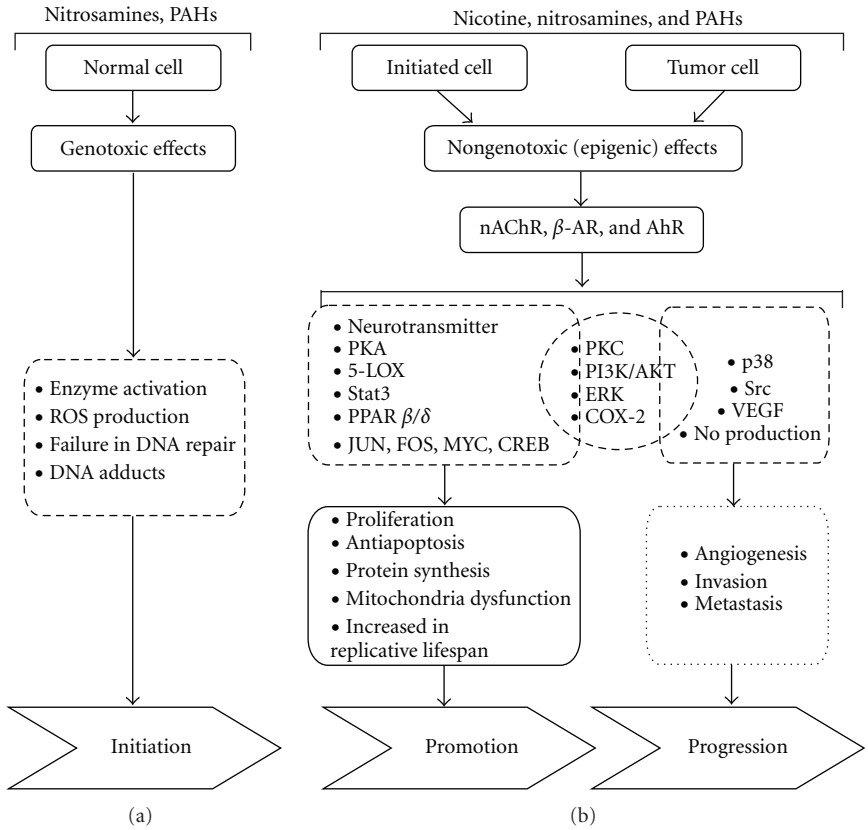


Figure 1: Diagrammatic models summarizing simplified molecular mechanisms of genotoxic and non-genotoxic modes of action in carcinogenesis by cigarette smoke. (a) Nitrosamines and PAHs are carcinogens, inducing genotoxic effects leading to cancer initiation. (b) Non-genotoxic (epigenic) effects of cigarette smoke components (nicotine, nitrosamines, and PAHs) in cancer promotion and progression. Activation of nAChR, $\beta$-AR, or AhR, followed by neurotransmitters release, activation of signaling pathways (PKA, 5-LOX, Stat3 and PPAR$\beta/\delta$), and increased the expression of transcriptional factors (JUN, FOS, MYC, and CREB) regulate cancer promotion by cigarette smoke. PKC, PI3K/AKT, ERK, and COX-2 signaling pathways downstream of receptors play important roles in both promotion and progression stages. p38, Src, VEGF, and NO releasing involve in enhancement of cancer progression by cigarette smoke.

## 5. Summary, Conclusive Remarks, and Future Perspectives

In this paper, we have reviewed the recent investigations concerning cigarette smoke and cancer development, promotion and progression. While chemicals with carcinogenic potentials in cigarette smoke are many (over 62), most research efforts have been devoted to three components of cigarette smoke: nicotine, NNK, and PAHs. While PAHs are common chemicals in the environment, nicotine and NNK are considered to be tobacco specific. These three important components of cigarette smoke, especially nicotine and NNK, therefore, are targeted as the major compounds of focus in this review. Many previous reviews have devoted to the interrelationship between cigarette smoke and lung carcinogenesis or the genotoxicity of cigarette smoke or its components. In this review, we are focused on the mechanistic information on tumorigenesis, especially those involving epigenetic or non-gentoxic effects. Aside from lung cancer, other tobacco-related cancers are also discussed. It is our hope that this review will summarize the vast information cumulated in the literature and provide valuable reference resource for those who are interested in tobacco-related carcinogenesis.

The overall mechanisms on carcinogenesis cancer promotion and progression are complex involving many molecular targets which include receptors, cell cycle regulators, mitogen-activated protein kinases, apoptosis mediators, angiogenic factors, and invasion, and metastasis mediators. Among the receptors, nAChR, $\beta$-AR, and AhR probably are the most important and have the closest association with cigarette smoke-induced carcinogenesis. Overexpression or activation of these receptors may result in the release of neurotransmitters and growth factors that participate in apoptosis inhibition, cell proliferation, angiogenesis, cancer cell invasion and metastasis. It should be noted that the importance of nAChR in cancer may be cell-type-dependent or specific and their sensitivity and expression can be also be modified by various environmental factors such as insecticide organophosphates [141].

As shown in Figure 1, signaling pathways, PI3K/AKT, Stat3, and ERK1/2 play important roles in the carcinogenesis processes. They are also common paths affected by the cigarette smoke components, including nicotine, NNK, and

PAHs. In addition, PKC, AKT, ERK, and COX-2 signaling pathways are involved in both promotion and progression stages by cigarette smoke. It is suggested that these molecules could be utilized the potential targets for future developments in cancer diagnoses or therapies.

Avoidance of cigarette smoke remains to be the best way of prevention for cigarette-related cancer. However, in view that tobacco smoke is legalized and smokers are still abundant, understanding on the health impacts by tobacco smoking still constitutes important public health concern. Understanding the disease process and the mechanisms involved is the first step to solution. The emerging understanding on the molecular mechanisms in the development and progression of caners induced by cigarette smoke provides novel inspirations and approaches for potential measures on early diagnosis, reduction in progression and metastasis, and therapy of cancers. Many dietary supplements, foods, or herbal medicines might significantly attenuate the proliferative effects by cigarette smoke. They may also enhance antigrowth signals to reduce cancer growth. From our own experience, the natural compound pterostilbene could induce apoptosis and autophagy in chemoresistant bladder cancer cells derived from nicotine exposure [142]. Future research on natural compounds may help to provide additional novel chemopreventive or chemotherapeutic possibilities in reducing cancer risks or other health impacts of cigarette smoke. This area of research is still weak and should be explored.

This review has also discussed the various molecular mechanisms and paths involved in carcinogenesis induced by cigarette smoke. However, there are still many mysteries in the carcinogenic process by cigarette smoke. Several recommendations can be offered for future research needs.

(1) In the past, most research efforts were focused on the proliferative and antiapoptosis mechanisms induced by cigarette smoking. As tumors are the results of multiple and interactive genetic abnormalities, study of cancers induced by cigarette smoke should assess more than one or two acquired alterations or paths. Explorations of other "paths" or mechanism other than those "popular" ones are needed.

(2) Those molecular pathways which are significantly activated by cigarette smoke are probably the most important ones involved in cigarette smoke-induced tumorigenesis. These pathways include nAChR signaling (such as $\alpha$7 nAChR, $\alpha$9 nAChR, or $\alpha$4/$\beta$2 nAChR), $\beta$-AR signaling, PI3K/AKT signaling, ERK1/2 signaling, Stat3 signaling, VEGF, and MMPs pathways, and so on. Targeting to modulate these pathways via dietary factors or therapeutic drugs may reduce cigarette smoking induced tumorigenesis significantly. Studies on the non-genotoxic (epigenetic) effects of cigarette smoke components are few and need more efforts. The epigenetic effects of cigarette component must be evaluated to include both upstream and downstream pathways.

(3) Carcinogenesis is often species or cell-type specific and can be influenced by many factors or cofactors. Proper study of carcinogenicity requires consideration of these different variables. The same factor which is highly oncogenic to certain cell type or individuals may not be oncogenic to others. Moreover, some cell type may become susceptible to a "carcinogen" only in the presence of certain factor(s), cofactor(s), genetic predisposition, or immune depression. Identification of such influencing factors will be important. Specific "mechanism" for carcinogenesis for the same "carcinogen" may also vary in different tissues. Information obtained will be helpful for future cancer prevention, diagnosis and treatment.

(4) Synergistic interaction between cigarette smoke components and other environmental toxicants or carcinogens, such as arsenic or dioxin, on cancer development has been demonstrated both epidemiologically and in animal studies [143–145]. Traditionally, most past investigations focused only on "single" compound or one cigarette smoke component. The synergistic interaction between otherwise "safe" level of environmental chemical and low level of cigarette smoke or its component (via either active or secondhand smoking) for carcinogenesis raised novel public health concerns and challenging questions. This area of research certainly deserves future attentions and efforts.

In conclusion, we have provided an overview on the major concepts and insights on the molecular mechanisms involved in cigarette smoke-induced cancers. It is hoped that these mechanistic insights can be translated into practical applications for the prevention and treatment of cigarette smoke-related cancers. We have also offered several recommendations for future research. We also hope that these suggestions will be helpful to those who are interested in this area of cancer research.

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*Review Article*

# Polonium and Lung Cancer

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The alpha-radioactive polonium 210 (Po-210) is one of the most powerful carcinogenic agents of tobacco smoke and is responsible for the histotype shift of lung cancer from squamous cell type to adenocarcinoma. According to several studies, the principal source of Po-210 is the fertilizers used in tobacco plants, which are rich in polyphosphates containing radio (Ra-226) and its decay products, lead 210 (Pb-210) and Po-210. Tobacco leaves accumulate Pb-210 and Po-210 through their trichomes, and Pb-210 decays into Po-210 over time. With the combustion of the cigarette smoke becomes radioactive and Pb-210 and Po-210 reach the bronchopulmonary apparatus, especially in bifurcations of segmental bronchi. In this place, combined with other agents, it will manifest its carcinogenic activity, especially in patients with compromised mucous-ciliary clearance. Various studies have confirmed that the radiological risk from Po-210 in a smoker of 20 cigarettes per day for a year is equivalent to the one deriving from 300 chest X-rays, with an autonomous oncogenic capability of 4 lung cancers per 10000 smokers. Po-210 can also be found in passive smoke, since part of Po-210 spreads in the surrounding environment during tobacco combustion. Tobacco manufacturers have been aware of the alpha-radioactivity presence in tobacco smoke since the sixties.

## 1. Introduction

WHO has declared a tobacco epidemic, indicating the spread of smoking dependency, which affects 1.3 billion people worldwide and results in 5.4 million tobacco-related deaths each year. If this trend continues, there will have been 10 million deaths by 2030 [1]. Smoking-related diseases include lung, esophagus, and pancreas cancer, cardiovascular diseases, COPD, pneumonia, sudden infant death syndrome, prematurity, and deaths caused by fires from cigarette stubs [2]. In Europe 650000 people die each year from smoking-related diseases.

Every year, approximately 11 million people are diagnosed with cancer worldwide; 8 million of them will die [3]. Cancer is a significant public health problem in Europe as well. In 2000, 1122000 deaths from cancer were registered in the 25 countries of the European Union (EU). From 1990–1994 to 2000–2004, mortality for all types of cancer in the EU declined from 185.2 to 168.0 per 100000 (world standard, −9%) in men and from 104.8 to 96.9 (−8%) in women [4, 5].

Tobacco smoking is a risk factor for six out of eight main death causes all over the world; with lung cancer being one of the six causes, tobacco represents the most important one [6, 7]. Each year 1.35 million new cases are diagnosed, which represents more than 12% of all the new cancer cases [8]. Furthermore, smoking is responsible for 1.18 million deaths from cancer (17.6% of the world total) [9], of which 21400 are lung cancers from second-hand smoking [10]. Survival rates for all stages and histological types are 10–15% [11].

Almost 46% of new cases of nonsmall-cell lung cancer pertain to the IIIB and IV stages [12]. In Europe, lung cancer mortality is 37.6 per 100000 people, ranging from a maximum in the UK (43.3 per 100000) to a minimum in Sweden (24.7 per 100000) [13]. In 2008, there were over 32000 new cases of lung cancer in Italy, 25147 of which

were men and 6955 women, while deaths reached 26211. Not so long ago, incidence was higher in men (at a 5 : 1 ratio), but nowadays it has dropped to half (2.5/1 ratio) [14]. This malignant tumor has become more and more frequent in women due to their increasing consumption of tobacco and higher predisposition to its carcinogenic effect. In fact, trends in incidence and mortality for both sexes have been completely different with declining rates in males and increasing ones in females. Despite this hecatomb of human lives, 1.3 billion people in the world and among them 47 million Americans (25%) [15] and 11.1 million Italians over 14 years (21.7% overall; 23.9% males and 19.7% females) continue smoking [16].

## 2. The Unrestricted Rise of Lung Cancer

Tobacco smoke has been known to be harmful for health since the fifties [17, 18]. In 1889, lung cancer was an extremely rare disease: only 140 cases were registered in the world [19, 20]. Notably, a recommendation was included in the first edition of the *Merck Manual*, which was published in 1889, suggesting that smoking could be used for the treatment of bronchitis and asthma [20].

In 1912, the very first cause/effect hypothesis between lung cancer and tobacco smoking was made in a research monograph by Adler [20, 21]. In the same year, surgeon Hugh Morriston Davies carried out the first pulmonary lobectomy for lung cancer in London. The patient died of infection 8 days after the operation, due to lack of cavity draining, a procedure not followed in such cases until 1929.

In 1914, Kellogg stated in a public health report that cancer killed 75000 people in the US each year, corresponding to 1 out of 20 deaths, and he noted that domestic animals were affected by cancer more frequently than humans, probably because of the indoor pollution deriving from combustions and tobacco smoking [22].

Almost two decades later, Dr. James Gilmore, a 48-year-old gynecologist from Pittsburgh, underwent the first successful left pneumonectomy for carcinoma. The operation was carried out by Dr. Evarts A. Graham, a pioneer in thoracic surgery [23–25]. Nearly 25 years later, Graham would die of the same disease that helped make him internationally renowned [26, 27].

Before the Second World War, experimental research on carcinogenesis from tar and polycyclic hydrocarbons was begun by an Argentinean researcher, Roffo [28–30]. Many of his studies were published in German scientific journals, which sank into oblivion after the war until WHO finally recognized him as the "forgotten father" of tobacco carcinogenesis, even though his research had already drawn the attention of tobacco manufactures in the past [31, 32].

On May 27, 1950, Ernest L. Wynder from the Sloan Kettering Institute and Evarts A. Graham published the first scientific paper on tobacco smoking as a possible etiological factor for bronchogenic carcinoma in *JAMA* [18]. 684 patients with lung cancer were studied, 96.5% of whom were heavy smokers while carcinoma was very rare (2.0%) in nonsmokers or light smokers. Wynder also assumed that 3-4 Benzopyrene, which was present in the cigarette smoke condensate, could cause cancer in humans. This hypothesis drove him to conduct the first experiments in tobacco smoke carcinogenesis.

In 1951, Richard Doll and Bradford Hill started the first extensive prospective epidemiological study, which was published in *British Medical Journal* in 1961 and confirmed the relationship between smoking and lung cancer [33]. The authors discovered that among the 1357 patients that were admitted to British hospitals with lung cancer, 99.5% were smokers.

A year later, *Reader's Digest*, which had a large circulation at the time, featured an article with the provocative title "Cancer by the Carton," in which the role of cigarette smoke in lung cancer was described as "*a medical controversy… largely kept from public notice*." [34] The article had an enormous impact on public opinion, putting pressure on the tobacco industry (Big Tobacco). As a consequence, on December 15, 1953, tobacco executives met at the Plaza Hotel in New York in order to create a cartel against the growing body of scientific evidence linking smoking to lung cancer, which had started to raise concern and distrust against tobacco manufacturers. Apart from the secret agreements, they jointly wrote the "*Frank Statement,*" which aimed at contrasting the evidence implicating smoking as a health issue [35]. This document/press release was published in more than 400 newspapers on January 4, 1954, reaching nearly 43 million readers.

A decade later, the first *Surgeon General's* report that addressed the consequences of tobacco smoke for public health was released [20]. By then, the distribution of free cigarettes at annual medical and public health meetings had already stopped.

The second part of the twentieth century saw a rapid increase in this disease leading to a lung cancer epidemic, especially in males of the developed countries [36, 37]. In the US, where measures for the control of tobacco dependency had already been established in the fifties, lung cancer incidence for men peaked in 1982 and a slow but steady reduction followed afterwards [38, 39]. Conversely, in other countries, where antismoking measures were less aggressive, a similar trend has not been observed and incidence has continued rising in some countries such as Japan [40–44].

## 3. What Does the Smoker Smoke?

Even though the carcinogenetic mechanisms of tobacco smoke are not fully explored [45], only very few smokers and non-smokers know what they inhale. Tobacco smoke is a mixture of a corpuscular part (5%) and a gas phase (95%). The former, without water or nicotine, is constituted of tar. There are 0.3–3.3 billion particles per milliliter of cigarette smoke and more than 4000 compounds [46, 47], including more than 60 agents with at least sufficient evidence of carcinogenicity in laboratory animals and 11 human carcinogens according to the International Agency for Research on Cancer (IARC) [48, 49].

Besides well-known organ-specific carcinogenic substances, such as polycyclic aromatic hydrocarbons, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK),2-Naphthylaine, 4-aminobiphenyl, arsenic, and chromium, there is another one, which has recently been involved in the spy case of Litvinenko: Polonium 210 (Po-210).

## 4. Chemistry

Polonium, also called "radium F," was discovered by Marie and Piere Curie in 1898 and was named after the home land of Curie-Sklodowska. For the discovery of radium and polonium Marie Curie received a Nobel Prize in Chemistry in 1911 [50, 51]. The element was discovered while they were investigating the cause of pitchblende's persistent radioactivity, even after the removal of uranium and radium. Their work was remarkable, considering the means available in the late nineteen century and the fact that the element can be found in uranium ores at about 0.1 mg per ton.

Polonium is a fairly volatile metal, rarely found in nature in pitchblende containing rocks, and constitutes $2.1 \times 10^{-4}$% of the Earth's crust [52]. The major resources of pitchblende are located in Canada, the US, Congo, and South Africa. Polonium has more than 30 radioisotopes, but Po-210 is the most dangerous and most frequent naturally occurring one [53]. This isotope has a half-life of 138.4 days, an effective biological half-time of 46 days [54], and can be created in the lab, when Bi-209 is bombarded with neutrons. It is a high energy $\alpha$-particle emitter (5.3 MeV), but it can also emit gamma photons with energy 803 keV and emission probability of nearly $1 \times 10^{-5}$ [55, 56]. It decays to stable Pb-206, and it has a melting point of 254°C and a boiling one of 962°C (for Pb-210 these temperatures are 327.5 and 1740°C resp.).

## 5. Toxicity

Polonium is a highly toxic element, with elevated specific radioactivity, and is dangerous to handle even in milligram amounts. The maximum allowable body burden for ingested Polonium is 1100 Bq, which is equivalent to a particle weighing only $6.6 \times 10^{-6}$ $\mu$g [57].

Alpha rays, which are formed by helium 4 (He-4) nucleus, are the least penetrating type of radiation and they manage to travel only a few centimeters in air. They can be easily stopped by obstacles, such as a sheet of paper, and they can penetrate living tissues by only a few microns [55, 58, 59]. In fact, since they lose all of their energy after a short distance, they can be dangerous for tissues only when substances emitting alpha particles enter the organism by respiration or ingestion.

In addition, alpha rays are highly ionizing and, therefore, are particularly harmful for living tissues. 1 mg of polonium can emit as many alpha particles as 5 grams of radium. The impact on humans can be devastating, as it can cause considerable damage by causing cell death, promoting a massive, progressive, and rapid necrosis, and not allowing the organism enough time to replace the quantity of dead cells [57].

## 6. Main Applications

Po-210 use is rather limited due to its high alpha radiation emissions and the difficult extraction process. The main uses are (a) as a resource of neutrons when it is mixed with beryllium, (b) as an energy resource for satellites and other space devices, (c) in antistatic devices of some precision instruments and in brushes that eliminate dust gathered on photographic film, and (d) in devices that eliminate static charges in textile mills, though less dangerous beta-ray sources are now more widely used [57].

## 7. From Earth to Tobacco

Traces of Po-210 can be found in many plants and foods and consequently, in human tissues as well [60, 61]. The principal resource of natural Po-210 is food. Spencer et al. report that 77.3% of the daily Po-210 intake of an adult male comes from food, 4.7% from water, and 0.6% from air. Notably, inhaling cigarette smoke can supply much more Po-210 (17.4%) than water and air combined [62]. 50–90% of the ingested Po-210 will promptly leave the body in feces, but the remaining fraction enters the blood circulation [63].

The discovery of the presence of Po-210 in tobacco smoke dates back to the early sixties, thanks to the work of Turner et al. [60], Marsden and Collins [64], and Radford and Hunt [65]. In fact, Po-210 and its precursor, lead 210 (Pb-210), are present in tobacco plants [66], as they may be absorbed in various associated ways.

(1) Through the plant's roots, directly from terrain that contains uranium [67–69].

(2) Coating on leaves as a result of meteorological events, rain, snow, and environmental dust. In fact, Radon-222, a product of U-238 decay, is a noble and volatile gas that can partially escape from terrain into the atmosphere and create Pb-210 and Po-210. These are absorbed by atmospheric dust, creating the Aitken particles that consequently lie on the leaves. The numerous trichomes of tobacco plants resemble filamentous pores and are metal accumulators, particularly of Pb-210 and Po-210. The quantity of the latter will then increase, as there is further Pb-210 decay [70, 71]. Fleischer and Parungo confirmed experimentally that radon and lead decay products are highly concentrated in the trichomes of leaves [71]. Additionally, accumulation mechanisms of Pb-210 on trichomes of tobacco have been widely discussed and studied by Martell and Poet [72, 73] while Skwarzec et al. suggested that this is the principal way Po-210 enters tobacco plants [68].

(3) On the other hand, the majority of authors, such as Singh and Nilekani, have identified the importance of the fertilizers employed [74]. Calcium polyphosphates fertilizers are enriched with radium, which is chemically similar to calcium, and derive from soil that contains pitchblende and apatite [67, 75]. Interestingly, according to several studies, Indian

cigarettes, which are made of scarcely fertilized tobacco, are 6 to 15 times less radioactive compared to the American ones, which derive from intensively fertilized plants [74].

## 8. From Tobacco to Lungs

The journey of Po-210 and Pb-210 towards bronchopulmonary apparatus starts by lighting a cigarette. In this combustion chamber, tobacco burns, reaching 800–900°C when inhaling, and smoke is created, which is composed of a corpuscular (5%) and a gas phase (95%) [46]. Po-210 and Pb-210 are adsorbed in the insoluble particles of the corpuscular phase [65]. The latter is present in a high quantity and is a weak alpha ($<1\times10^{-5}$), gamma, beta, and X emitter. All these inhaled particles are deposited in the broncho-pulmonary apparatus and particularly in segmental bronchi bifurcations, due to ciliary action. According to measurements by Cohen et al., radium and thorium are also present in cigarettes; however, 99% of the radioactivity comes from Po-210 [75], which remains in the broncho-pulmonary apparatus after inhalation [76].

All these particles have a different "destiny" based on the efficacy of the mucous-ciliary clearance. This mechanical purification is reduced gradually in smokers with COPD, resulting in the accumulation of insoluble Pb-210 particles, which decay to Po-210 over time [70, 77]. In fact, the more severe COPD becomes, the greater the risk of radioactive load accumulation is [77].

Subsequently, radioactive particles reach various organs and tissues through pulmonary and systemic circulation and cause mutations of the genetic cellular structure, deviations of the standard cellular characteristics, accelerated ageing, and quicker death due to a wide range of diseases [78, 79]. In smokers, Po-210 levels are in fact significantly higher in blood (by 30%) [65, 80], urine (6-times higher) [81], liver, kidney, heart, and psoas muscle [82]. Little and McGandy estimated that Po-210 concentration in blood is 63.64 mBq/kg of blood in smokers and 28.12 mBq/kg of blood in non-smokers [83]. Notably, concentrations of Pb-210 and Po-210 in rib bones and alveolar lung tissues were two-times higher in ex-smokers compared to non-smokers, even a year after smoking cessation [66].

Polonium radiation in the bronchial epithelium depends not only on the particle concentration of these areas, but also on the time of their permanence. Half-life of polonium is 138.38 days and of lead 22 years, which decays afterwards into polonium. There is a significant cancer risk due to chronic exposure to low levels of insoluble alpha-emitting particles [84, 85], which are responsible for high radiation doses in small tissue areas particularly in the bifurcations (hot spots) [70]. This process is facilitated by the above-mentioned impaired mucous-ciliary clearance of smokers. In fact, according to Auerbach et al. [86], metaplastic lesions are present in the ciliated epithelium of all heavy smokers [87, 88]. Po-210 of the insoluble particles becomes even more penetrative because of zones with damaged or scarcely ciliated epithelium, where mucous mainly stagnates [65, 89]. More and more studies suggest that smokers and ex-smokers with moderate to severe COPD have a higher incidence of lung cancer [77, 90–92].

## 9. Po-210 Quantity in Tobacco Smoke

Po-210 alpha radioactivity in tobacco smoke depends on several variables: geographic region of tobacco growth, storage time and modality, presence of a filter, its length and composition, and the way of smoking [85]. Furthermore, the associated risk of smoke derives not only from the quantity and quality of carcinogenic substances, but also from the scarce efficacy of filters, which fail to reduce their amount adequately. In fact, common filters, found in the cigarettes of commerce, are able to reduce Po-210 activity on average by 4.6% [93]. There is evidence that resin filters may reduce lung exposure to alpha radiation even more [94].

Radford and Hunt [65] and Mussalo-Rauhamaa and Jaakkola [95] reported that about 6.5% to 22% of the Po-201 contained in cigarettes was found in mainstream smoke. Other authors stated different percentages, ranging from 3.7% to 58% [96]. According to Parfenov, approximately 50% of a cigarette's Po-210 is transferred with the smoke, 35% remains in the stub, and 15% is found in the ash [97].

Professor Gattavecchia from the Complex Unit of the Institute of Chemical, Radiochemical, and Metallurgic Sciences of University of Bologna (SMETEC), in association with ENEA (Italian National Agency for New Technologies, Energy, and Sustainable Economic Development) and the Italian Society of Tobaccology (SITAB), have conducted various studies on the alpha radioactivity of Po-210 in tobacco smoke. It has been confirmed that a cigarette with tobacco of Western origin emits 75 mBq of alpha radioactivity from Po-210, distributed in mainstream (5 mBq, 6.7%), sidestream (1.2 mBq, 1.6%), and ash (68.8 mBq, 91.7%) [97–100].

## 10. Po-210 and Second-Hand Smoking

Many studies have already reported that second-hand smoke is an important risk factor for lung cancer. After studying 91540 people for 14 years, in 1981 Hirayama demonstrated the lung cancer mortality of non-smoker wives or husbands was one-third higher compared to those with non-smoker partners [101].

This increased risk was also confirmed by a vast analysis of two case-control studies conducted in the US and Europe, in which a dose-response relationship between lung cancer risk and prolonged exposure to second-hand smoking has been found among partners, in workplaces and in public places. Risk for one-off exposure to spousal smoking increased by 18% (95% CI = 1–37%) and for long-term exposure by 23% (95% CI = 1–51%). Augmented risk for long-term exposure was also found for the work place (OR = 1.25; 95% CI = 1.03–1.51) and public places (OR = 1.26; 95% CI = 1.01–1.58) [102].

It should be considered that passive smokers are exposed to the same components as active smokers, including

radioactive elements. As a matter of fact, Po-210 in second-hand smoke is 50–70% the quantity found in active smoke. Moreover, passive smokers are exposed to environmental pollution from radon, as well as from Po-210 of cigarette smoke, both of which increase lung cancer risk [87, 103, 104].

## 11. Po-210 and Narghil Smoke

Po-210 is also present in narghilé smoke. An international multidisciplinary team (from Egypt, Arabia, and France), coordinated by Khater et al., has recently published a pioneering study on narghilé (shisha, hookah) tobacco radioactivity [105]. Before this research, only very few data were available on this issue [106, 107].

The research was based on the measurement of some natural radionuclides activity and the estimation of the internal radiation dose due to narghile tobamel (moassel) smoking. Tobamel is a fashionable flavoured tobacco-molasses mixture (with added glycerol) currently used in narghilé. However, there are other forms such as jurak, similar to tobamel, but unflavoured, containing minced fruits and no glycerol [105]. It is also much stronger in nicotine. The results of the study revealed a wide range of radioactivity concentrations (in Bq/kg dry weight): U-238 = 55 Bq (19–93), Th-234 = 11 Bq (3–23), Ra-226 = 3 (1.2–8), Pb-210 = 14 Bq (3–29), Po-210 = 13 Bq (7–32), Th-232 = 7 Bq (4–10), and K-40 = 719 Bq (437–1044). The researchers concluded that the average concentrations of natural radionuclides in moassel tobacco pastes were comparable to their concentration in Greek cigarettes and tobacco leaves, and lower than that of Brazilian tobacco leaves [105].

Another recent study on the radioactivity of Greek tobacco leaves used for cigarettes showed that the annual effective dose due to inhalation by adult smokers varied from 42.5 to 178.6 $\mu$Sv/y (average 79.7 $\mu$Sv/y) for Ra226; 19.3 to 116.0 $\mu$Sv/y (average 67.1 $\mu$Sv/y) for Ra-228; 47.0 to 134.9 $\mu$Sv/y (average 104.7 $\mu$Sv/y) for Pb-210. In sum, the order of magnitude was the same for each radionuclide. The sum of effective doses of the three radionuclides varied from 151.9 to 401.3 $\mu$Sv/y (average 251.5 $\mu$Sv/y). Notably, the annual effective dose from Cs137 of Chernobyl origin was three orders of magnitude lower as it varied from 70.4 to 410.4 nSv/y (average 199.3 nSv/y) [108].

The results of Khater et al., found that the radioactivity concentration in tobacco products basically depends on the tobacco content itself, not on other ingredients such as molasses, glycerol, or fruits. Interestingly, the lower yield of Po-210 in jurak might be in relation with the Indian origin of this smoking paste. The reason might be that Po-210 alpha-radioactivity of Indian tobacco would be several times lower than that of Western tobacco [74].

## 12. Po-210 Carcinogenicity in Tobacco Smoke

Eighty-five to ninety out of a hundred lung cancers are caused by tobacco smoke; nevertheless, less than 20% of smokers get lung cancer [7]. If individuals contracting cancer on exposure to cigarette smoke are identified, the information can certainly be incorporated into effective prevention strategies [109].

Many factors could influence individual susceptibility to lung cancer in smokers. Polonium is among them, albeit it is still less considered or even ignored as a carcinogenic substance, which is also due to years of concealing by tobacco manufacturers [110]. As a matter of fact, when associated to other mutagenic and carcinogenic nonradioactive substances, which are inhaled with tobacco smoke (such as aromatic hydrocarbons, cadmium, and N-nitrosamine) [111], it seems to constitute the principal etiological factor for lung cancer [112], as long-term tissue exposure to alpha radiation can induce cancer either by itself or in association with other non-radioactive carcinogenic substances.

Polonium 210 emits alpha particles, which have a penetration limit of about 40 microns or less in animal tissue, but a very high damaging effect [55, 58, 59]. Since the late nineties, IARC has identified Po-210 as a carcinogenic element for laboratory animals, classifying it among the Group 1 agents [49].

DNA chromosome damage by exposure to alpha radiation is 100-times greater than the one caused by other types of radiation [113]. Little and Radford estimated that the radiation dose of the bronchial epithelium of bifurcations in the inferior lobes of people smoking for 25 years would be 2 Sv [114]. This can be explained by the local accumulation of Pb-210 insoluble particles [72]. According to Martell, the cumulative dose of alpha radiation in bronchial bifurcations of smokers that die of lung cancer is approximately 16 Sv (80 rad). This dose is sufficient to induce a malignant transformation caused by alpha-particles interaction with basal cells [115, 116].

Black and Bretthauer reported that Po-210 radiation dose in heavy smokers was up to 82.5 mrad (0.83 mSv) per day [117]. Radford and Hunt, estimated that the radiation dose for a person smoking two packs of cigarettes a day may be up to 0.4 Sv a year or 10 Sv over a 25-year period [65]. Such a radiation exposure dose rate was about 150-times higher than the approximately 0.05 Sv per 25 years received from natural background radiation sources.

Many lung cancers are adenocarcinomas, a type of lung cancer that Po-210 inhalation can induce in laboratory animals [116]. Kennedy et al., induced lung cancer in hamsters, histologically similar to bronchoalveolar carcinomas (BAC) of humans, after Po-210 intratracheal instillation [118]. They also implicated the bronchiolar cell of Clara as the origin of these tumors. Moreover, according to Marmorstein, adenocarcinomas could be induced with as little as 15 rad of radioactive polonium, corresponding to one-fifth of the dose inhaled by smokers of two packets per day over a 25-year period [113].

Boffetta et al. recently reviewed seven case-control studies and estimated that the odds ratio of BAC for smoking at all was 2.47 (95% CI = 2.08–2.93). The authors also reported that the risk increased linearly with duration, amount, and cumulative cigarette smoking and persisted long after smoking cessation [119].

*12.1. Mechanism of Action.* In a recent study, Prueitt et al. tried to explain the way alpha radiations affect DNA [120]. Ionizing radiation, including Po-210, could silence the tumor suppressor gene p16(INK4a) by promoter methylation. Inactivation of this gene was found in lung cancers of both smokers and radiation-exposed non-smoker workers. The authors concluded that such inactivation was shown to play a major role in carcinogenesis, but further studies could demonstrate the level of this role compared to other carcinogenic substances.

*12.2. Biological Harm.* But what is the level of biological damage caused by tobacco smoke Po-210? Estimating the damage is a very difficult and complicated task. Using the 1990 ENEA data on the average time of Po-210 presence in lungs, which is 53 days [121], the data of the BEIR IV Committee on lung cancer risk after exposure to radon and its decay products (Pb-210, Po-210) [122], and the data of the International Commission on Radiological Protection (ICRP), which are based on the survivors of the bomb A of Hiroshima [123], it is possible to estimate the lung cancer risk, which is $4 \times 10^{-4}$ year$^{-1}$ (4 cases per 10000 smokers per year, which corresponds to nearly 5000 cases for the 11.1 million Italian smokers). This estimate does not take into account the promoter role of Po-210 (cocarcinogen) in the bronchopulmonary cancer and the overall carcinogenic activity of all substances [124].

To render the biological harm deriving from Po-210 in smoke more comprehensible, it has been compared to the damage caused by radiation in conventional chest X-rays. Since the dose of a modern chest radiograph is 0.034 mSv [125, 126], a smoker of 20 cigarettes per day receives a radiation dose of 0.08–0.09 Sv equivalent to approximately 300 chest X-rays per year [98, 99, 113, 127]. However, the alpha radioactivity alone does not cause the steep rise of the carcinogenic risk; instead, it is the combined and multiplicative action of each carcinogenic and co-carcinogenic component responsible for such consequence [88, 111, 128].

## 13. A Histotype Shift

There is evidence that in the last 40 years a histotype change of lung cancers has been noticed, shifting from squamous cell carcinoma to adenocarcinoma, in which the bronchial-alveolar (BAC) subtype is also included [129]. The above-mentioned shift was observed in the early seventies and has been noted ever since in the US [130, 131] and Europe [132].

The factors that have induced this shift are various and perhaps not all known. Nevertheless, almost all of them are linked to the tobacco cultivation and cigarette manufacture changes since the fifties. The most common are as follows.

(a) The utilization of different varieties of tobacco in the US cigarette blends. This change reduced benzopyrenes in smoke, but produced an increase of nearly 50% in nicotine-derived nitrosaminoketone (NNK) in the last quarter of the twentieth century [133, 134].

(b) The introduction of low-tar, low-nicotine, filtered cigarettes since the mid fifties, which seems to have contributed to the overall decline in lung cancer and the upward trend in the incidence of adenocarcinoma [135–138]. Some studies demonstrated a decline in lung cancer risk in smokers of filter cigarettes [139]. Even the common filters made of cellulose acetate have contributed to the aforementioned histotype change [140], nevertheless, smokers frequently breath in these cigarettes more deeply and as a result, a greater quantity of carcinogens is transported more distally, towards the smaller bronchial airways, where adenocarcinomas often arise [141, 142]. In addition, the increased consumption of filtered cigarettes has also reduced the yield of carcinogenic polycyclic aromatic hydrocarbons (PAHs), which are inducers of squamous cell carcinomas, simultaneously increasing the carcinogenic tobacco-specific N-nitrosamines (TSNAs), which are inducers of adenocarcinomas [137, 143].

Interestingly, in the fifties the race for the safer filter led to a severely dangerous incident. Lorillard produced 13 billion cigarettes from 1952 through 1956 based on a filter composed of asbestos and cotton fibers. Each filter contained 10 mg of crocidolite, the fibers of which could be found in the mainstream smoke from the first two puffs. Consequently, a person smoking a 20-cigarette pack each day could inhale more than 131 million crocidolite fibers, which were longer than 5 $\mu$m, in a year's time [144]. When proof of the danger of asbestos started to surface, these cigarettes were called in, after spreading harmful fibers to the lungs of thousands of smokers.

(c) The massive introduction of polyphosphate fertilizers in tobacco cultivations, contributing alpha radiation (from Pb-210 and Po-210) and TSNAs significantly [48], especially in Western cigarettes rather than in the ones from poor agricultural areas like India. Studies showed that American tobacco is 5.5-times more radioactive compared to the Indian-grown one (19.09 mBq/g versus 3.33 mBq/g resp.), due to the polyphosphate fertilizers [74, 145]. Because of the lower radioactivity, the prevailing type of lung cancer histotype in India is the squamous cell carcinoma and the cell type patterns have remained unchanged virtually since the early sixties [146]. As a matter of fact, in 1962, Viswanathan et al. reported 50.5% squamous cell carcinomas versus 28.4% adenocarcinomas [147] while more recent studies reported 58–67% versus 10–19%, respectively, [148–150].

## 14. How to Reduce the Radioactive Load of Tobacco Smoke?

Regulating and reducing this harmful radiation, which comes from fertilizers, could help reduce lung cancer incidence [151]. Tobacco radiation could be reduced by applying various solutions, which may also work combined.

(a) Use of alternative polyphosphate sources, such as organic fertilizers from animals [151].

(b) Use of ammonium phosphate as a fertilizer, instead of calcium phosphate [151].

(c) Different storage methods. A study proved that Po-210 radioactivity of tobacco rose over time while in storage [152]. As a consequence, harvesting tobacco while it is still green and avoiding prolonged storage in silos in order to prevent an increase in Po-210 concentration due to Pb-210 slow decay could be recommended.

(d) Genetic modifications of tobacco plants with significant reduction of trichomes concentration on the leaves, on which Pb-210 and Po-210 accumulate [71].

(e) Resin filters may decrease lung exposure to alpha radiation [94]. On the contrary, common filters reduce Po-210 activity, on average, by 4.6% [93].

(f) LaRock et al. recommended a biological way to remove Po-210 by treating polyphosphate rocks with bacteria capable of reducing sulphates [153].

(g) Perhaps the simplest and most applicable solutions would be the quantitative decrease in polyphosphates use in tobacco cultivations and the regulation of the maximum acceptable level of alpha radiation of cigarettes, which should also be clearly indicated on the packet [110].

## 15. Big Tobacco Has Been Aware but Kept Quiet

While multinational tobacco manufacturers have been aware of the alpha-radioactivity presence in tobacco smoke since the sixties, they have covered it up strategically. Not by chance, the polonium dossier was symbolically entitled "*waking a sleeping giant*" [110].

Among the 37 million documents that were released through the site www.pmdocs.com, one can find the lawsuit of the State of Minnesota against Philip Morris Incorporated, et al., in which there are 481 confidential documents and memorandums on the alpha-radioactivity from Po-210 in tobacco smoke (still available on 1 January 2011). The archives bring out the fact that Philip Morris has been aware of the lead and polonium existence in cigarettes since the sixties [154], as was also proved from studies by Turner et al. 1958 [60], Radford and Hunt (1964) [65], and recently by researchers from Mayo Clinic [110]. In these internal documents, it can be seen that there was a clear interest in polonium's radioactivity and the induction of bronchogenic carcinomas in laboratory animals and presumably in humans [155]. In fact, there was a recommendation to avoid any public attention to the problem for fear of "*waking a sleeping giant*" [110].

In 1980, one confidential memorandum revealed that the issue was mainly caused by calcium phosphates fertilizers employed in tobacco cultivations. Moreover, cigarette manufacturers knew about the studies conducted by Martell [72], regarding the possibility of decreasing tobacco and smoke radioactivity by using ammonium phosphate instead of calcium phosphate as fertilizer. Nevertheless, this recommendation was considered to be "an expensive point" [152].

So far, the majority of public opinion still ignores the presence of polonium radioactivity in tobacco smoke and the serious public health threat that it represents. Yet, from a communicative and motivational point of view, it could become a great opportunity for prevention and smoking cessation. For now, it seems that something has changed in the media and scientific world since the widely covered Litvinenko case and the paper of Muggli et al. [110]. In fact, the authors of the aforementioned study and the Italian Tobaccology Society have already requested the placement of a clear indication about the radioactivity content on cigarette packets.

## 16. Conclusions

Polonium-210 represents one of the principal causes of lung cancer and its shift from squamous cell carcinoma to adenocarcinoma. Provided that it is true that tobacco manufacturers have been aware of the presence of Po-210 in smoke since the early sixties and concealed its existence intentionally in various ways, it is likely that the medical and scientific sector is guilty of having ignored it.

It is necessary that the medical and scientific world becomes aware and conscious of this problem, creating systematic educational programs of tobaccology in the university curricula of the medical sciences courses. Likewise, governments should force manufacturers to introduce cigarettes with low Po-210 concentration and place a clear indication about this on the packet in order to reduce smokers' risk.

Finally, since people fear everything that is radioactive, perhaps it would be useful to create an adequate information campaign so as to enable and accelerate smokers' motivational pathways and increase the efficacy of anti-smoking programs [156].

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*Review Article*

# Cigarette Smoke, Bacteria, Mold, Microbial Toxins, and Chronic Lung Inflammation

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Chronic inflammation associated with cigarette smoke fosters malignant transformation and tumor cell proliferation and promotes certain nonneoplastic pulmonary diseases. The question arises as to whether chronic inflammation and/or colonization of the airway can be attributed, at least in part, to tobacco-associated microbes (bacteria, fungi, and spores) and/or microbial toxins (endotoxins and mycotoxins) in tobacco. To address this question, a literature search of documents in various databases was performed. The databases included PubMed, Legacy Tobacco Documents Library, and US Patents. This investigation documents that tobacco companies have identified and quantified bacteria, fungi, and microbial toxins at harvest, throughout fermentation, and during storage. Also characterized was the microbial flora of diverse smoking and smokeless tobacco articles. Evidence-based health concerns expressed in investigations of microbes and microbial toxins in cigarettes, cigarette smoke, and smokeless tobacco products are reasonable; they warrant review by regulatory authorities and, if necessary, additional investigation to address scientific gaps.

## 1. Introduction: Chemical and Biological Components of Tobacco and Smoke

For many years, scientists have undertaken studies to define the chemical composition of green tobacco leaf, cured-fermented-stored tobacco leaf, and tobacco smoke with the intent of identifying chemicals that may pose a significant health risk [1–4]. An illustration has been prepared of the annual increase, from 1954 to 2005, in the total number of tobacco smoke chemicals that have been identified [4]. Today, there is a consensus of opinion that cigarette smoke consists of at least 5,300 different chemicals [4]. These chemicals are present in the complex aerosol that consists of a heterogeneous mixture of gas- (vapor-) phase and particulate- ("tar-") phase components [1–4].

Detailed listings of the chemicals in mainstream and sidestream tobacco smoke are available, and an assessment of their propensity for harm has been presented; a partial listing of references is included [1–4]. Most of the chemicals, toxicants, and carcinogens in tobacco smoke arise from the burning (pyrolysis) of the tobacco [1, 2, 4]. The potential for harm has also been studied for chemicals that do not arise from the burning of tobacco. The chemicals include metallic and nonmetallic elements, isotopes, and salts [1, 2, 4]. In addition, pesticides and other intact agrochemicals have been identified in tobacco smoke [1, 2, 4]. Also included in this tabulation of chemicals in smoke are menthol and flavorants [4].

In 1985, Hoffmann and coworkers, who had studied the chemical composition of tobacco smoke for many years, began formulating a list of chemicals that were designated as biologically active, carcinogenic, cocarcinogenic, or tumorgenic, reviewed previously in [4]. The tabulation was revised and became the basis for the list of "*Hoffmann Analytes*" [4]. In 1985, different working groups met to identify those chemicals in tobacco smoke that are most likely to be

carcinogenic to humans as defined by criteria of the International Association for Research on Cancer (IARC), an intergovernmental agency forming part of the World Health Organization, and by the US National Toxicology Program (NTP) [1, 2, 4].

## 2. The Changing Cigarette

The identification, classification, and concentration of the various chemicals in cigarette smoke have been challenged by changes in the design of cigarettes. A comprehensive review of "The Changing Cigarette" was published by D. Hoffmann and I. Hoffmann in 1997 [5].

Subsequently, other investigators addressed changes in cigarettes and their potential for risk [6–12]. By way of example, a partial tabulation of changes in cigarette includes (a) increased cigarette length (85 mm king sized and extra long "120's") and, for some brands, reduced circumference (23 mm "slim" cigarettes), (b) variation in the blend of natural tobaccos of diverse types, country of origin, and curing processes, relative percent tobacco leaf (lamina) versus tobacco ribs/stems, and tobacco weight per rod, (c) incorporation of manmade tobacco, sometimes referred to as reconstituted or "sheet" tobacco, (d) introduction of additives to the tobacco (casings) that include diverse flavorings (licorice and honey), humectants to retain tobacco moisture, and menthol to ameliorate smoke irritation and promote smoking acceptance by youngsters and "starters" (e) addition of ammonia, to facilitate "freebasing" the nicotine to enhance the pharmacological effect (impact), (f) application of diverse glues and printing ink, (g) configuration of diverse cigarette filter materials (cellulose acetate, paper, or combination of both), (h) alteration of filters with charcoal and schemes whether the carbon was dispersed throughout the filter plug or retained in a filter cavity, (i) variation in filter design (filter length, fiber packing/crimping, fiber density, and filter ventilation) to effect tar delivery (full flavor cigarettes versus ultralight low-tar cigarettes), (j) paper type, paper porosity, with burn accelerators to promote burning, or with modifications to reduce the propensity for sustained burning and affect a "fire safe" designation, and (k) diverse methodologies to reduce "tar" and nicotine yields in mainstream smoke of cigarettes that have been smoked mechanically [6–12].

The topic of "*The Changing Cigarette*" has been addressed and summarized in a recent report of the Surgeon General entitled "How Tobacco Smoke Causes Disease" [13]. A review of the scientific and medical literature has shown that (a) changing cigarette designs over the last five decades, including the introduction of cigarette filters and low-tar cigarettes, have not reduced overall disease risk among smokers and may have hindered prevention and cessation efforts, (b) there is insufficient evidence that novel tobacco products reduce individual and population health risks, and (c) the introduction of novel tobacco products that are marketed as reduced-risk cigarettes may encourage tobacco use among youngsters. These changes have challenged tobacco policy and regulation [13].

## 3. Tobacco and Harm Associated with Microbes

Our review of the aforementioned writings [1–4] and many other related reports, addressing chemicals in tobacco smoke of cigarettes have shown that the writings do not address the propensity for harm that may be associated with microbial elements of smokeless and smoking tobacco articles. A partial listing of tobacco-associated microbial elements include bacteria (Gram positive and Gram negative), bacterial spores, fungi (yeast and mold), fungal spores, cell wall components (certain glucans and flagellum), and diverse microbial toxins that include exotoxins and endotoxins. Examples of bacterial-derived toxins include endotoxins (lipopolysaccharide, LPS; inflammatory factor) and fungal-derived mycotoxins (aflatoxins, AF type B1; human carcinogen) [1–4].

There exists today a concern of the potential health risks associated with diverse microbial elements that are known to exist in smoking and smokeless tobacco products that are currently being marketed. This subject has not been addressed in the context of national tobacco control policy or regulatory authorities.

Harm is to be recognized as persistent or chronic inflammation. Inflammation is mediated by different leukocyte subsets and different secreted factors (Figure 1). Inflammation not only establishes a microenvironment that fosters the malignant transformation and tumor growth but also promotes microbial colonization.

## 4. Research Objectives

The goal of this paper is to profile the scientific and medical literature addressing microbes in tobacco with the intent to determine whether there is sufficient evidence to warrant additional investigations to assess propensity for human harm. The impetus for undertaking this work was derived in part from the fact that several teams of investigators, including our own, have published observations during the last few years that suggest microbial elements maybe harmful to tobacco users.

Notable in a first analysis of the literature on the microbiology of tobacco we discovered that there were few recent reports (1990 to 2010) in peer-reviewed, mainstream, scientific and medical journals by scientists of tobacco companies. By way of example, Philip Morris has contracted the Life Science Research Office, Inc., (LSRO, Bethesda, MD), to identify methods to evaluate tobacco products and with a particular focus on identifying research schemes and assays for assessing reduced-risk tobacco articles [14]. Three monographs published by LSRO in 2007 detailed the chemicals to be assayed and recommended procedures. The subject of microbial flora and microbial toxins was not addressed, nor were schemes and methodologies for the assessment of tobacco associated bacteria, mold, or microbial toxins [14].

Therefore, the question arose as to whether the issue of health risks associated with microbial elements in smokeless and smoking tobacco was not investigated by laboratory scientists working at the tobacco companies or whether the subject was studied and the information withheld as private

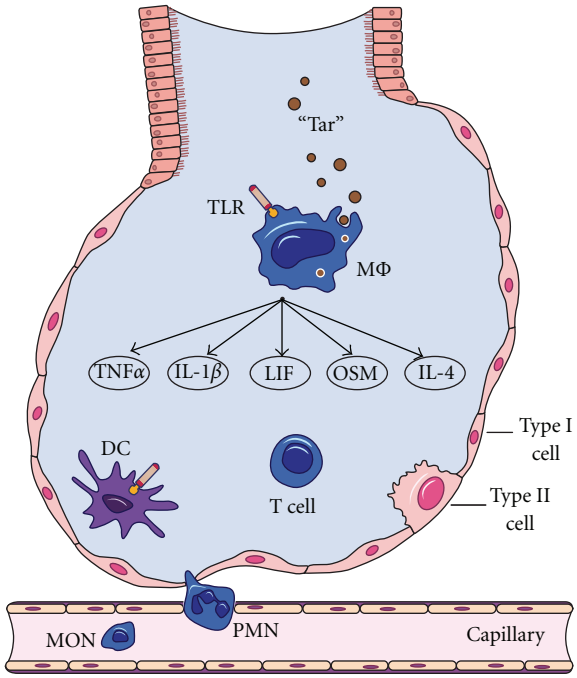


Figure 1: A schematic view of an alveolus that depicts the effect of inhaled tobacco smoke on the terminal (respiratory) structure of the lung. Particulate matter "Tar" in tobacco smoke is inhaled deep into the lung where it is recognized by macrophages. The macrophages arise from the blood monocytes that migrate into the lung where they undergo differentiation and maturation. Macrophage phagocytosis of the chemical-rich "Tar" evokes the production of diverse proinflammatory mediators (for details, see Figure 1). Macrophages have toll-like receptors (TLR) that recognize diverse microbes and toxins (LPS is recognized by TLR-4). Shown in this illustration is the production of five proinflammatory cytokines: tumor necrosis factor, type alpha (TNF$\alpha$), interleukin 1-beta (IL-1$\beta$), leukemia inhibitory factor (LIF), oncostatin M (OSM), and Interleukin-4 (IL-4). These soluble factors interact with other cells of the lung, and the response of these cells is thought to accelerate, amplify, and prolong pulmonary inflammation. The target cells may include T cells. The T cell that is depicted herein is representative of many different T cell subsets, including T helper cell subsets Th1, Th2, and Th17. Type I epithelial cells are the major cells lining the alveolar space, and facilitating $O_2/CO_2$. The type I cells are spread out and cover about 90 to 95% of the alveolar surface. The type II cells form only 5 to 10% of the surface but produce surfactant proteins. Polymorphonuclear leukocytes (PMN) mediate inflammation in multiple ways, including the production of an oxidative burst. Dendritic cells (DC) are professional antigen-presenting cells; they also mediate inflammation.

and confidential. The paucity of the literature on health risks associated with microbes in smokeless and smoking tobacco is to be contrasted to the numerous reports by tobacco scientists researching other health-related issues, such as potential reduced-risk exposure tobacco products (PREPS) [15].

## 5. Perspective and Limitations

The authors are immunologists and have an active research interest in addressing tobacco-associated chronic pulmonary inflammation. It is acknowledged that immunological responses and inflammation would not be a primary interest by other investigators whose primary interests are in the disciplines of microbiology/metagenomics, aerosol-associated inhalation toxicology, infectious diseases, and clinical pathology (oral and lung). Also, the work presented herein is limited in scope. The authors retrieved numerous documents from databases, but space restrictions permit citing but a few of the writings. Also, many of the writings were internal documents and were not subjected to peer-review. Some documents cited are old and are addressed herein to provide a historical perspective. Lastly, the documents are fragmented and it is recognized that conflicting findings and interpretations may be presented by competing tobacco companies.

## 6. Literature Search

A computer-based structured search of the literature was conducted. The study scheme included a search of the literature from PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Scopus (http://www.scopus.com/home.url). Also, included was a search of Google (http://www.google.com/). A search was also made of patents in the database of the US Patent and Trade Office (http://www.uspto.gov/). In addition, searches were made for documents that were released by the tobacco companies and made public as a consequence of the tobacco Master Settlement Agreement. To this end, we searched database records of over 11 million documents in the digital archive that were established and which are maintained currently at the University of California, San Francisco (http://legacy.library.ucsf.edu/). We also searched the database from Tobacco Documents (http://tobaccodocuments.org/).

The searches were performed using conventional telegram-style search short-string text formulations with Boolean operators as described in PubMed. Illustrative key search words were bacteria, mold, fungi, yeast, tobacco, smoke, endotoxin, mycotoxin, cured, fermented, lipopolysaccharide, aflatoxin, and microbiology. We also used unique search words, such as author's name, project designation, report codes, cigarette brands, and Bates number. The references cited in the retrieved literature were reviewed to identify other topic-specific writings Table 1.

## 7. Tobacco-Associated Chronic Inflammation

Chronic inflammation is associated with malignant transformation, tumor growth, and, possibly, tumor metastasis, reviewed in [44–52]. Examples of the association of cancer with chronic inflammation include (a) lung cancer and cigarette smoke (aerosol), (b) malignant mesothelioma and asbestos (fibers), (c) stomach cancer and *H. Pylor*i (bacteria), (d) malignant melanoma and ultraviolet sun light (irradiation), (e) liver cancer and aflatoxin (mycotoxin), and (f) cancer of the uterine cervix and human papilloma virus. Thus, malignancy at diverse body sites, and of various tissues, is associated with chronic inflammation provoked by assorted

Table 1: History of investigations of microbes and microbial toxins in tobacco and tobacco products.

| | |
|---|---|
| 1896 [16] | Results are reported for studies that were undertaken to characterize the microbes of tobacco before and during tobacco fermentation. |
| 1899 [17] | German bacteriologist H. E. Suchsland announces that the delicate aroma and subtle shades of flavor which affect the palate of the smoker are not due to the tobacco but are attributed to the microbes which aid in the process of tobacco fermentation. A patent based upon this observation was submitted, presumably to improve the poor quality of German tobacco by adding to the harvested tobacco leaves bacteria that he had isolated and grown in his laboratory from high-quality West Indian tobacco. |
| 1954 [18] | The microbial degradation of nicotine and nicotinic acid was reported. The morphological and physiological properties of the nicotine-decomposing bacteria were also described. |
| 1955 [19] | W. C. Flanders of R. J. Reynolds Tobacco Company issues a 70-page report of a three-year study to determine if the number of microorganisms (bacteria and mold) changed appreciably during aging. Experiments were also conducted to determine if the recorded changes in the microbes follow the changes in the chemical components of tobaccos. These studies were continued and extended for several years. |
| 1957 [20] | *Pseudomonas aeruginosa* and other potentially pathogenic fungi and bacteria were identified in snuff. Similar microbial isolates from a patient was the basis for the physician to theorize that some of the snuff-derived microbes may be responsible in part for chronic bronchitis. |
| 1958 [21, 22] | The results of studies were reported that had been undertaken to characterize the deposition of cigarette smoke particles and debris released from the cigarette filter into the human respiratory tract. Popular brand cigarettes were smoked mechanically and in a manner to reflect normal smoking behavior. The studies documented that tobacco flakes and fine tobacco leaf debris were released into mainstream smoke from the cigarette filter of all brands that were tested (Tareyton, Winston, Kent, L&M, Marlboro, and Viceroy). The tobacco flakes and other particulates (filter fibers and carbon from charcoal filters) were studied by light and electron microscopy. |
| 1966 [23] | Toxic fungi were identified in tobaccos. |
| 1967 [24, 25] | Comparative studies were preformed for microbiological activity in the smoke of popular brand nonfiltered and filtered cigarettes that had been "cold smoked" or lit. Viable bacteria were found in the smoke of all cigarettes tested. |
| 1972 [26] | The tobacco from different popular brands of cigarettes was analyzed for bacteria. The number of bacteria was determined on "our own" (Philip Morris) and competitive cigarette fillers. This test was run for several months and each month Viceroy, Brown & Williamson's product, always showed the lowest degree of "contaminant." The difference between the brands was statistically significant. Brands tested included Salem, Pall Mall, Chesterfield, Kool, Kent, Viceroy, Winston, and Marlboro. The number of bacteria on Marlboro were "too numerous to count." |
| 1972 [27] | A 189-page report was prepared by investigators at the Brown & Williamson Tobacco Company that presents methods for the microbiological examination of tobacco and tobacco products. The writings include the description of techniques for the quantitative determination of bacteria and fungi and methods for the isolation of potentially human pathogenic microorganisms including Coliform bacteria. Also identified were *Staphylococcus aureus*, *Enterococci*, *Pseudomonas*, *Clostridium*, and *Aspergillus*. |
| 1972 [28] | A 52-page report that describes a "contact plate method" in which a whole cigarette is rolled over the surface of the nutrient agar dish. Viable microbes that are transferred from the cigarette to the plate are illustrated. Presumably, the intent of the assay was to measure the growth of microbes that would be transferred from the cigarette paper to the hand of the smoker. Other studies showed the growth of microbes from a natural wrapper of a cigar. Also, culture methods were established for testing for coliform bacteria and for counting viable fungi in tobacco. |
| 1972 [29] | A 346-page in-house document is produced by the British-American Tobacco Company entitled "Methods for the Microbiological Examination of Tobacco and Tobacco Products." The authors describe the "Public Health Aspects" of smoking and smokeless tobacco products. They note that "[T]he detection of micro-organisms of health significance in tobacco products must be expected to be regarded as undesirable or even unacceptable by public agencies, regardless of whether there is proof of the significance in initiating or spreading infection in man. Therefore, it is suggested that tobacco products should be substantially free, or contain only minimal numbers, of micro-organisms of potential health significance to man which could conceivably occur on tobacco…" Suggested standards are presented for tobacco products for various bacteria and fungi, and standards that had been established for food products (fish, sausage, meat pies, cream yogurt, soft cheese, and pasteurized milk). |
| 1991 [30] | Philip Morris characterizes the microbial population on Marlboro tobaccos throughout the processing line. Five different Marlboro Make-Your-Own tobaccos with various anti-microbial preservatives were evaluated microbiologically for mold and bacteria over time. The microflora of Marlboro raw and tobacco blends were defined for burley, oriental, flue-cured, and other tobacco types. |

Table 1: Continued.

| | |
|---|---|
| 1992 [31] | Bacillus spores were identified in chewing tobacco sold in the USA. Broth of the culture microbes evoked plasma exudation from the oral mucosa when tested using a hamster cheek pouch assay. |
| 1995 [32] | In an oral presentation, Hasday describes for the first time the presence of endotoxin in cigarette smoke. |
| 1990 [33] | Scientist from Imperial tobacco (Canada) report the development of an easy-to-search database on the microbes associated with tobacco. |
| 1999 [34] | Bacterial endotoxin was identified as an active component of cigarette smoke. |
| 2004 [35] | A US Patent was awarded for a method and system for assay and removal of harmful toxins during the processing of tobacco products. |
| 2004 [36] | Microbiologists in Sweden used a mass-spectrophotometry-based assay to document that tobacco smoking increased dramatically the air concentrations of endotoxin (LPS). The authors note that smoke-derived LPS may be a health risk factor associated with environmental tobacco smoke. |
| 2004 [37] | A US Patent was assigned to Philip Morris for an "antibacterial lavage" method to treat tobacco leaves so as to eliminate or reduce bacterial endotoxins (LPS) and tobacco-specific nitrosamines that are formed during the curing process. Bacteria found on tobacco leaves were reported to be primarily Gram-negative bacteria, including pseudomonades and enterobacters. In the awarded patent, Hempling notes that bacterial endotoxins can remain as a residue on the tobacco even after the bacteria have been destroyed. |
| 2004 [36] | The microbiological composition of tobacco products was defined using culture and chemical analysis. Tobacco smoke was analyzed chemically, and LPS was measured for tobacco leaves and cigarette tobacco. |
| 2005 [38] | US Military publishes a report of an investigation that documents bacterial species diversity of varying brands of cigarettes made in the Middle East that were thought to be associated with illnesses of American soldiers deployed in Operation Iraqi Freedom. |
| 2006 [39] | Cigarette smoke was identified as the source of elevated levels of endotoxin (LPS) found in indoor air. |
| 2007 [40] | Identification of microflora on tobacco using culture-independent methods based on the amplification of microbial 16S rDNA sequences directly from the leaf surfaces. The investigators discovered also that three of five dominant bacterial species on the tobacco could not be cultivated. |
| 2008 [41] | The microbiological composition of tobacco products was defined using culture and chemical analysis (of tobacco leaves) or chemical analysis only (tobacco and tobacco smoke). Mesophilic bacteria dominated among the bacteria in both fresh and cured tobacco leaves; however, a wide range of other bacteria, including Gram-negative bacteria, and fungi were delineated. Microbial flora was compared in studies of tobacco from cigarettes from different countries. LPS was also measured. |
| 2008 [42] | Bacteria grown from a single flake of tobacco from all brands of smoking (cigarette, cigar, and pipe) and smokeless (snus, snuff, and long cut) tobacco products. In many instances, the bacteria from the tobacco caused hemolysis of blood in blood agar and liquid broth cultures. |
| 2010 [43] | Twenty-seven species of bacteria were identified in an analysis of both unaged tobacco and flue-cured tobacco by 16S rRNA sequence analysis. More species ($N = 23$) were identified from the unaged flue-cured tobacco leaves than in the aging leaves ($N = 15$ species). |
| 2010 [43] | Fifteen classes of bacteria and a broad range of potentially pathogenic organisms were detected in all cigarette samples studied. In greater than 90% of the tobacco samples, the investigators identified *Acinetobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, *Klebsiella*, *Pseudomonas aeruginosa*, and *Serratia*. The bacteria were identified using a 16S rRNA-based taxonomic microarray. Cloning and sequencing were used to evaluate total bacterial diversity of four brands of cigarettes. Previous studies have shown that smoking was associated with colonization by pathogenic bacteria and an increased risk of lung infection. This study, however, was the first to show that cigarettes themselves could be the source of exposure to a wide array of potentially pathogenic microbes. |

items that include smoke, bacteria, fibers, irradiation, toxins, and viruses.

## 8. Cigarette Smoke, Chronic Inflammation, and Impaired Immunity

Cigarette smoke is known to induce chronic inflammation of the lung [53–60]. More recently, a substantial body of information has been obtained to suggest that long-term cigarette smoking may not only have an adverse effect of systemic immunity but also skews both innate and adaptive immune responses [61–65].

## 9. Study Rationale: Evidence-Based Health Risks of Tobacco-Associated Microbes

Concern has been expressed by many investigators that microorganisms on cured tobacco might represent a health risk.

By way of example, in 1968, Wood [66], a scientist at the British American Tobacco Company, wrote a 37-page report addressing the possible transfer of viable microorganisms into mainstream smoke. In this internal document, he notes that cured tobacco, of various types, has long been known to contain bacterial spores. Likewise, Wood [66] and others [23] have addressed the possibility that tobacco-associated mold may also represent a health hazard to smokers. Support for this concern was derived in part from a paper published in *Science* by Forgacs and Carll two years previously in which they reported the identification of toxic fungi in tobacco [23]. In the *Science* paper, the investigators exposed mice to smoke from fungally contaminated hay. The mice developed pulmonary emphysema and other pathological conditions; in contrast, mice exposed to smoke from sterile, uninoculated hay remained normal clinically. In a letter to the Associate Scientific Director of the Council for Tobacco Research, dated 1964, Forgacs, with more than 16 years of research experience as a mycologist, states that he had examined mycologically a number of tobacco products, including cigarettes that had been purchased on the open market [67]. Forgacs observed that the tobacco of all cigarettes contained fungal mycelia and spores [67]. In part, the origin of his health concern is based upon the knowledge of (a) widespread fungal contamination of tobacco products, (b) heat stability of the mycotoxins; (c) known animal toxicity, (d) reasonable assumption that some of the fungi are carcinogenic, and (e) potency at low doses, see also [68].

Wood argues that

> "[W]hile it is quite impossible to deduce, from this (mouse) experiment, the likely effect of smoke from a cigarette containing fungally contaminated tobacco, the implications are sufficiently important to warrant some consideration of the role which micro-organisms may play with regard to smoke toxicity. For instance, it is possible that viable spores might be transferred to mainstream smoke and thus enter the lungs; pathogenic species, even in small numbers, could clearly have harmful effects, while very large number of otherwise harmless micro-organisms might lead to a significant concentration of genetic material. Alternatively, during the vegetative stage of their residence on tobacco the micro-organisms might produce toxins which could transfer direct to smoke or metabolites which on burning could give toxic smoke constituents."

The report by Wood also describes some preliminary experiments which were undertaken to show whether bacterial or fungal spores could transfer into tobacco smoke. Two schemes were used to trap the cigarette smoke; these were a test tube bubbler and a micropore filter. These samples from the bubbler and the filter were tested for the growth of microorganisms. Growth of microbes was observed; however, technical problems were encountered including poor reproducibility and smoke toxicity. The results were inconclusive. Our search for subsequent studies by Wood addressing this subject failed to identify subsequent experiments or published reports. Studies by Slutzker et al. were negative [69]. In 1967, Curby reported to The Council for Tobacco Research the results of comparative studies that he had undertaken to determine the microbiological activity in the smoke from filter and nonfilter cigarettes. Different popular brands of cigarettes were obtained from local vendors in Brookline, Mass, USA. Comparative analyses were made of bacteria released from cigarettes that had been "cold smoked" (not lit) or smoked in the usual manner (lit). The tobacco smoke collection system was tested for sterility by means of conventional microbiology culture procedure and by means of electronic analyses of particle size and number. Viable bacteria were identified in the smoke from all cigarettes tested. The number of liberated organisms was much greater when the cigarette was burning [24, 25].

Before profiling more recent studies, a brief overview is warranted of what many internal documents of the tobacco industry have entitled the "*Microbiology of Tobacco.*"

## 10. Microbiology of Tobacco

The "*Microbiology of Tobacco*" has been the focus of many studies. It was not surprising to learn from our paper that most of all the major tobacco companies have studied this issue for many years. Listed below are varying topics addressing bacteria, mold, and mycotoxins in tobacco and references

(a) chemical and microbiological changes during curing [16, 19, 70–75],

(b) bacteria in cigarettes; product comparison (also, see below) [17, 76–79],

(c) databases of tobacco microbes [33, 40, 80],

(d) tobacco microbe control [81],

(e) microflora community of tobacco [82–88],

(f) quantitative studies of tobacco microflora [89–91],

(g) growth of mold in stored tobacco [26, 92],

(h) growth of *Aspergillus* from tobacco [93–95],

(i) microbial degradation of nicotine [18, 96],

(j) examination of cigarettes from mold-damaged and nondamaged tobacco [97],

(k) isolation of viable fungi from snuff [98],

(l) sterilization/treatment to remove NNK [37, 99–105],

(m) removal of harmful toxins on tobacco [35, 95],

(n) inhibiting mycotoxin production [106],

(o) microbiology of cigarettes, pipes, cigars, and snuff [27–30, 107–111].

From about the early 1970s, extensive research was conducted on the *Microbiology of Tobacco.* Many reports reflected the interest of the major tobacco companies. These studies sought to identify different bacteria and molds and to

count the number of colony-forming units (CFU) during processing. The number of bacteria and molds present in green, freshly harvested tobacco was compared to that of various stages of curing, fermentation, and long-term storage. In many cases, more than one million bacteria were found in a gram of tobacco (a 100 mm cigarette has about 0.9 grams of tobacco). Comparative studies included various types of tobacco (Bright and Burley) and different curing methods (field versus flue cured). In these studies, profiles were established for leaves of the different types of tobacco that had been picked from various positions of the plant. Diverse environmental conditions were evaluated, and these included variations in temperature and moisture. Analyses were made of the number of bacteria in popular brand cigarettes. In many instances, the number of bacteria of a particular company's brand was compared to brands marketed by competitors. In addition to cigarettes, studies were performed for cigars and snuff. Considerable effort was devoted to defining procedures for the sterilization of tobacco to reduce or prevent the growth of mold. The methods used included (a) washing methods using various solutions (bleach), (b) irradiation with microwave, ultraviolet light, and gamma radiation, (c) exposure to various gases, and (d) treatment with different antibacterial and antifungal agents (antibiotics). One scheme was to destroy all of the bacteria on freshly harvested green tobacco leaves and then seed the leaves for fermentation using selected colonies from in-house batch-scale production. Quality control of the tobacco was important as high levels of mold produced an unacceptable "off-taste."

## 11. Pathogenic Bacteria of Chewing Tobacco

Studies have been conducted by investigators of the tobacco industry (see above) and health community to address the potential of bacteria, molds, yeast, and microbial toxins found in different types of smokeless tobacco (snuff, snus, and long cut) [20, 26, 31, 43, 112, 113].

In 1951, Dynert published in *The New England Journal of Medicine* a case report of a patient with chronic bronchitis. *Pseudomonas aeruginosa,* often colonized in COPD patients, and a few colonies of *Staphylococcus aureus* were identified in bacteriological examinations of the subject's sputum [20]. The patient used snuff, and it was theorized that the snuff may have been the source of the pathogens. A study was then undertaken of 22 samples of previously unopened packs of snuff. The following microorganisms were grown from more than 50% of the snuff samples: *Bacillus rubitilles*, *Staphylococcus aureus* (coagulase positive), *Staphylococcus albus* (coagulase positive), *Pseudomonas aeruginosa*, *Staphylococcus aureus* (coagulase negative), and *Staphylococcus albus* (coagulase negative).

In 1991, Varma reported the isolation of nine species of *Aspergillus* in stored leaves of chewing tobacco [112]. Approximately 18 of the Aspergilli were found to be mycotoxigenic. All aflatoxigenic strains of *A. flavus* produced aflatoxin $B_1$. Patulin and ochratoxin were produced by *A. ochraceus*. Sterigmatocystin was produced by three different strains.

Warke [103] studied the microbiological quality of chewable, often sweet, tobacco mixes known as "Gutka" used by millions of children and adults in India where it is made and often exported. Of the 15 samples studied, all contained aflatoxins $B_1$, $B_2$, and $G_2$. Samples exposed to $^{60}$Co radiation displayed a marked reduction of viable CFU. Sterilization of tobacco in the manufacturing has been described in US Patents [105].

In 1992, Rubenstein reported the identification of large number ($>10^6$ CFU) of a *Bacillus* species in chewing tobacco sold in the USA [31]. Supernatants of the cultured bacteria evoked a plasma exudate in studies in which the supernatant was instilled into an intact hamster cheek pouch.

## 12. Pathogenic Bacteria of Cigarettes

Some bacteria grow in unique microenvironments, and some are difficult to grow using traditional broth- and agar-based methods. This technical difficulty may also apply to growing bacteria that have adapted to unique conditions that develop during the curing and fermentation of tobacco. Accordingly, it is believed that conventional methods may not accurately define the microflora of diverse tobacco products [43, 113]. Consequently, there may be an incomplete understanding of the bacterial diversity in the tobacco of cigarettes and also the impact these microbes and microbial toxins may impose on the smoker [113].

Recently, the bacterial metagenomic of cigarettes were characterized using a 16S rRNA-based taxonomic microassay as well as traditional cloning and sequencing methods. The brands included Camel, Marlboro, Kool, and Lucky Strike. The results of this study showed that the number of microorganisms in cigarettes may be as vast as the number of chemicals in these products. Fifteen classes of bacteria were identified [113]. Particularly noteworthy was the identification of a broad range of potentially pathogenic microorganisms detected. More than 90% of the tobacco samples from the cigarettes contained *Actinetobacter*, *Bacillus*, *Burkholderia*, *Closteridium*, *Klebsiella*, *Pseudomonas aerogenosa*, and *Serratia*. Other bacteria that are known to be potentially pathogenic to humans and detected using the metagenomic technology were *Campylobacter*, *Enterococcus*, *Proteus*, and *Staphylococcus* [113].

Reported also in 2010 were the results of an investigation of the diversities of unaged and flue-cured tobacco leaves using a 16S rRNA sequence analysis scheme [43].

Others have reported the identification of potentially pathogenic bacteria in commercial cigarettes. One study was undertaken to assess the bacterial diversity of cigarettes that were thought to be linked to severe pneumonitis in US military personnel deployed in Operation Iraqi Freedom [38]. Eight species of *Bacillus*, including five new species, and one new species of *Kurthia* were isolated from the cigarettes. Some of these species have been identified elsewhere to cause hypersensitivity pneumonitis and other respiratory syndromes [38]. This study was of particular interest to many because the cigarettes were made in Iraq and not manufactured by a major tobacco company. Undertaking this

investigation, the question arose as to whether the cigarettes that had been purchased by soldiers from street vendors had been intentionally altered by adding pathogenic bacteria and/or mold. This theory was disproven.

Another study was conducted by a group of investigators in Sweden who characterized the bacterial and fungal community in warehouse tobacco [41].

We have reported previously the establishment of a novel bioassay which showed that bacteria were grown routinely from a single flake of tobacco that had been placed on the surface of a sheep blood agar plate [42]. Of eight popular brands of cigarettes, bacteria grew from almost all (>90%) of the flakes. Similarly, bacteria were grown from a single flake, and also with a high frequency, from tobacco that had been retrieved from cigar filler and from smokeless tobacco (snus, snuff, and long cut). Some bacteria induced hemolysis of the blood in the agar dishes. The destruction of the red blood cells was readily visible as a yellow zone surrounding a single tobacco flake. Expanding studies documented the hemolysis of human blood in agar or nutrient broth cultures. Thus, as discussed later, bacteria could be carried deep into the respiratory tract by a single tobacco flake sucked from the cut surface of a cigarette filter and transported into the bolus of smoke that is inhaled deep into the lung. A single tobacco flake may be envisioned as a matrix for delivering diverse bacteria into the respiratory tract of an immunologically compromised long-term smoker.

## 13. Cigarettes with Mold

Mold has been identified in the tobacco of popular brand cigarettes, and concern has been raised as to the propensity of these microbes as a health risk to the smoker. Presented herein is a partial listing of papers that have identified mold in cigarettes [78, 114–116] and in marijuana [116].

As early as 1971, Papavassiliou and coworkers concluded that "[C]igarettes are contaminated with various fungi." They studied cigarettes that were manufactured in the USA, Canada, England, France, Belgium, Germany, Jordan, and Egypt. Hundreds of strains of fungi were isolated. The Greek scientists demonstrate that the most prominent fungi were *Aspergillus* (28 strains from Greek cigarettes and 35 strains from other countries). They raised the question as of the association of the fungi with allergies but commented that this issue has not been resolved [114].

In 1983, Kurup and colleagues reported the identification of allergenic fungi in smoking materials and discussed the health implications of their findings [115]. Concern has been expressed as to the health risks associated with mold in cigarettes.

Writing in the Journal of the American Medical Association, Verweij et al. addressed the propensity of heath risks associated with fungal contaminates of tobacco and marijuana [116]. They concluded that "[A]ll cigarette brands tested ($N = 14$ brands) had some degree of fungal contamination, although not every cigarette was found to have a positive culture."

## 14. Transfer of Tobacco Flake to Mainstream Smoke

The filter of a cigarette is often contaminated with loose tobacco flakes, tobacco fines, and tobacco dust. In one examination, the filters of 11 brands of cigarettes were examined in freshly opened packs. For all brands, cigarettes were observed with tobacco flakes on the filter. Examination of the filters with the naked eye showed that 127 of 208 (61.1%) of the filter had tobacco particles [42]. The release of tobacco flakes into mainstream smoke has been described previously [21, 22].

The tobacco flakes that contaminate the filter arise from tobacco that escapes from the nonfilter, sometimes called the distal, end of the cigarette. Most probably the flakes are jarred loose during manufacturing, shipment, and daily transportation, especially in a pack in which more than one-half of the cigarettes have been used [117, 118].

The release of flakes from the cut surface can readily be demonstrated by comparing the cut surface of the filter before and after smoking the first puff. The single flake may be viewed as a matrix for carrying bacterial and fungal agents in mainstream tobacco smoke. Thus, the burning of the tobacco during cigarette smoking does not exclude the exposure to tobacco-associated microbes and microbial toxins.

Bacteria are also released from the barrel of the cigarette. This was demonstrated in investigations in which a cigarette was rolled over the surface of a nutrient agar dish.

## 15. Endotoxin (LPS) in Mainstream and Sidestream Tobacco Smoke

In 1999, Hasday and his colleagues reported the identification of bacterial endotoxin as an active component in cigarette tobacco and cigarette smoke [34]. The authors showed that the dose of LPS delivered from smoking one pack of cigarettes was comparable to that of the LPS that had been previously shown to be associated with adverse health effects in cotton textile workers. With the knowledge that LPS is one of the most potent inflammation-inducing agents, the work by Hasday attracted considerable attention, reviewed in [32]. In 2004, Larsson et al. reported that they were able to demonstrate unequivocally that high levels of LPS are inhaled during active cigarette smoking and, more importantly, that environmental tobacco smoke may involve inhalation of amounts of endotoxin that are dramatically greater than those existing in indoor environments free from tobacco smoke [36]. In 2006, these findings were confirmed and extended [39]. Particularly notable is that studies of Larsson and colleagues used a mass-spectrometry-based assay that circumvents the problems often associated with the biologically based LPS assay.

## 16. Analysis of Findings and Policy Recommendations

The results of this literature review have documented that the tobacco microflora has been the subject of many studies by

investigators of tobacco industry and academic communities. During the last 50 years, there has been an imbalance, however, in the attention devoted to addressing the identification and propensity of the harm of tobacco- and tobacco-smoke-associated chemicals and in the attention devoted to characterizing microbes and microbial-derived factors.

Ample information has accumulated to suggest that microbes and microbial-derived factors may contribute to the health risks of smoking and smokeless tobacco products. Moreover, the microbes may facilitate microbial colonization of the mouth and airway, the induction of chronic inflammation through the activation of diverse leukocyte subsets, alteration of the tissue microenvironment, and microbial-toxin-induced pathologies. The current health concerns recently expressed by investigators of various disciplines, and with different research interests, in peer-reviewed published research articles are reasonable and validate that additional investigation of the microbiology of tobacco is warranted. The findings reported herein relate to National Tobacco Control Policy and specifically FDA Regulation of Tobacco Products [119].

Based upon the information obtained in this paper, we recommend the following for consideration and possible regulatory action.

(1) Tobacco products should be assessed with the knowledge that they contain bacteria, mold, and microbial toxins.

   (a) In this context, the designation of tobacco products is to include conventional and novel products that contain tobacco, including items which are smoking and smokeless tobacco articles.

   (b) National and international registries of known human carcinogens should not be used as the sole criteria for assessing tobacco-associated human health risks. Any and all tobacco-associated agents that induce any human pathology should be included in risk assessments.

   (c) Tobacco in smoking and smokeless tobacco articles should be assessed for their propensity to induce chronic inflammation. Chronic inflammation is known to be induced by diverse bacteria (Gram positive and Gram negative) and fungi, living or dead, whole or fragmented, and intracellular and membrane components. Chronic inflammation is known also to be induced by diverse toxins of bacteria and/or fungi including, but not limited to, endotoxins, exotoxins, and mycotoxins.

   (d) Chronic inflammation associated with bacteria, fungi, and microbial toxins of tobacco products should include inflammation of any and all target sites, including tissues of the mouth, nasopharynx, and lung.

   (e) In addition to chronic inflammation, harm of microbial elements of tobacco should be assessed in the context of other known tobacco-associated diseases, including chronic obstructive pulmonary disease, asthma, bronchitis, and alveolar hypersensitivity.

(2) Tobacco-specific nitrosamines (NNK) are human carcinogens that are present in mainstream smoke, sidestream smoke, and smokeless products. NNKs arise primarily from the microbial degradation of nicotine in tobacco. Different technologies have proven effective in preventing the formation of NNKs. It is recommended that these technologies be implemented and that guidelines for tobacco articles be established for reduced NNK-products.

(3) The criteria, protocols, and procedures used by the FDA in the assessment of harm associated with mycotoxins in food products should be applied to loose leaf tobacco, smoking tobacco products, and smokeless tobacco articles. Mycotoxin action levels should be established to provide an adequate margin of safety to protect human tobacco users.

## Abbreviations

AFL-$B_{1:}$ Aflatoxin, type $B_1$
CFU: Colony forming unit
DC: Dendritic cell
IARC: International Association for Research of Cancer
IL-1$\beta$: Interleukin-1, beta
IL-4: Interleukin-4
LIF: Leukemia inhibitory factor
LPS: Lipopolysaccharide
LSRO: Life Science Research Organization
LTDL: Legacy Tobacco Document Library
MON: Monocyte
NTP: National Toxicology Program
OSM: Oncostatin M
$PGE_{2:}$ Prostaglandin E2
PMN: Polymorphonuclear leukocyte
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
TLR: Toll-like receptor
TNF$\alpha$: Tumor necrosis factor, alpha.

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*Research Article*

# Molecular Mechanisms of Cigarette Smoke-Induced Proliferation of Lung Cells and Prevention by Vitamin C

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Lung cancer is the leading cause of cancer dearth. Cigarette smoking is the strongest risk factor for developing lung cancer, which is conceivably initiated by proliferation. Here, we show that low concentration of aqueous extract of cigarette smoke (AECS) causes excessive proliferation of human lung epithelial cells (A549) without any apoptotic cell death. The causative factor responsible for AECS-induced proliferation has been identified as p-benzoquinone (p-BQ). Coimmunoprecipitation and immunoblot experiments indicate that p-BQ binds with epidermal growth factor receptor (EGFR). However, in contrast to EGF, it causes aberrant phosphorylation of EGFR that lacks c-Cbl-mediated ubiquitination and degradation resulting in persistent activation of EGFR. This is followed by activation of Hras + Kras and the downstream survival and proliferative signaling molecules Akt and ERK1/2, as well as the nuclear transcription factors c-Myc and c-Fos. Vitamin C and/or antibody to p-BQ prevents AECS/p-BQ-induced proliferation of lung cells apparently by inactivating p-BQ and thereby preventing activation of EGFR and the downstream signaling molecules. The results suggest that vitamin C and/or antibody to p-BQ may provide a novel intervention for preventing initiation of lung cancer in smokers.

## 1. Introduction

Lung cancer is the leading cause of cancer death in the United States and throughout the world [1]. Cigarette smoking is the strongest risk factor for developing lung cancer. Smoking and exposure to environmental tobacco smoke account for 90% of lung cancer cases, and smokers have a 20-fold increased risk of death from lung cancer compared to nonsmokers [2]. However, the carcinogenic mechanisms of tobacco smoking are not well understood [3]. The most significant property of cancer cells is that they undergo excessive proliferation. Lung cancer arises after a series of progressive pathologic changes (preneoplastic lesions) that are initiated by proliferation (hyperplasia) [4]. In almost all instances, unregulated cell proliferation together with suppressed apoptosis constitutes the minimal common platform upon which all neoplastic progression occurs [5]. It has been proposed that increased proliferative activity is causally linked to carcinogenesis and tumor progression [6]. Experimental and theoretical support for the hypothesis that increased proliferation itself is a contributory factor to carcinogenesis stems mainly from studies with chemical carcinogens in rodent tumor models and mathematical modeling of tumor progression [7]. Clinical observations also suggest a possible contributory role of increased cell proliferation to genesis and/or progression of human cancers [7]. Since cigarette smoke (CS) causes lung cancer, it is expected that CS should promote cell division. In fact, preliminary observations indicate that hyperproliferation of cells occurs in response to smoke exposure [8–10]. However, the molecular mechanisms of CS-induced cell proliferation are yet to be known. This is particularly because cigarette smoke (CS) is a highly complex mixture containing about 4000 compounds, including carcinogens, free radicals, and long-lived radicals such as semiquinones [11, 12]. It is a conjecture whether one particular compound or a number of compounds in CS are responsible for proliferation of cells. We have isolated a major semiquinone from CS and characterized it as p-benzosemiquinone (p-BSQ) [13, 14]. p-BSQ is present in substantial amounts (100–200 $\mu$g/cigarette) in smoke from all commercial cigarettes examined as well as

Kentucky research cigarettes [15]. p-BSQ causes cytotoxicity and tissue damage through conversion to p-benzoquinone (p-BQ), which occurs by disproportionation and oxidation by transition metal-containing proteins [14, 16]. Here, we show that CS-induced proliferation of lung cells is completely prevented by antibody to p-BQ and that p-BQ in amounts derived from CS mimics CS-induced lung cell pro-liferation. Overexpression and/or hyperactivity of the epidermal growth factor receptor (EGFR), accompanied by several downstream cytoplasmic signal transducers, including Ras-MAPK cascade as well as the cell survivor factor Akt (protein kinase B) has been shown to play a causal role in the proliferation and progression of lung tumors [4]. Lemjabbar et al. observed that CS-induced cell proliferation was accompanied by phosphorylation (activation) of the epidermal growth factor receptor (EGFR) [9]. Abdelmohsen et al. showed that p-BQ induced activation of extracellular signal-regulated kinase ERK1/2 via the activation of EGFR [17]. Here, we demonstrate that p-BQ, apparently derived from p-BSQ of aqueous extract of CS (AECS), is responsible for AECS-induced cell proliferation via activation of EGFR, Ras, ERK1/2, and Akt as well as the transcription factors c-Myc and c-Fos. p-BQ is strongly inactivated by vitamin C, and earlier we had reported that CS-induced cytotoxicity and lung damage is prevented by vitamin C [18–22]. Here, we show that both antibody to p-BQ and vitamin C prevent AECS/p-BQ-induced proliferation of human lung cells apparently by inactivating p-BQ.

## 2. Materials and Methods

*2.1. Chemicals and Reagents.* p-Benzoquinone (p-BQ) was procured from Merck and freshly crystallized before use. Benzo[a]pyrene (BP) was obtained from Fluka. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) were obtained from Toronto Research Chemicals Inc. Tyrphostin AG1478 was purchased from Sigma-Aldrich, USA. The "In Situ Cell Proliferation Kit, FLUOS" was obtained from Roche Applied Science, Germany. The Annexin V-FITC kit was purchased from BD Biosciences. The kit for protein estimation was purchased from Bio-Rad, USA and the Protein A-Sepharose CL-42 beads from GE Healthcare, USA. The chemiluminescence kit for immunoblot analysis was procured from Cell Signaling Technology, USA. All other chemicals were of analytical grade. The antibody to p-BQ, raised in rabbit after immunization with p-BQ-bovine serum conjugate, was supplied by Abexome Biosciences, Bangalore, India.

*2.2. Cigarettes.* All the experiments were performed using cellulose-acetate filter-tipped Kentucky reference cigarettes (3R4F) obtained from the University of Kentucky, College of Agriculture Reference Cigarette Program, Lexington, Kentucky, USA.

*2.3. Preparation of Aqueous Extract of Cigarette Smoke (AECS) Solution.* The method of preparation of AECS was so devised as to simulate the manner in which the respiratory tract lining fluid is exposed to CS during the process of smoking by humans [18]. Smoke from one cigarette was extracted with 1 mL of 50 mM potassium phosphate buffer, pH 7.4, filtered through 0.22 $\mu$m Millipore filter and the pH adjusted to 7.4. The aqueous extract of CS (AECS) solution thus obtained was used immediately.

*2.4. Measurement of p-Benzoquinone (p-BQ).* p-BQ was measured by HPLC as described before [14]. The column used was a LichroCART 350-4, RP-18 (5 $\mu$m) (Merck). p-BQ was detected at 245 nm at the retention time of 4.75 min using a mobile solvent of methanol: water (90 : 10 v/v) at a flow rate of 0.5 mL/min. The limit of detection was 500 pg.

*2.5. Cell Culture.* A549 human lung carcinoma cells were maintained in Hams F12 medium (GIBCO-BRL, USA) containing 10% fetal calf serum (GIBCO-BRL, USA), 100 units/mL penicillin, 100 $\mu$g/mL streptomycin, and 4 mM glutamine/mL. L132 (normal human lung epithelial cells), Vero (African green monkey kidney cell line), and HepG2 (human liver cell line) cells were maintained in Dulbecco's Modified Eagle Medium (GIBCO-BRL, USA), containing 10% fetal calf serum (GIBCO-BRL, USA), 100 units/mL penicillin, 100 $\mu$g/mL streptomycin, and 4 mM glutamine/mL. All the cells were obtained from National Centre for Cell Sciences (NCCS Pune, India). The cells were grown at 37°C in a humified incubator maintained in an atmosphere of 95% air and 5% $CO_2$.

*2.6. Cytotoxicity Assay.* The cytotoxicity of AECS, p-BQ, NNN, NNK, and BP was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described earlier [15]. Briefly, after treatment with different compounds, the culture medium was replaced with serum-free medium containing 0.5 mg/mL MTT, and cultures were incubated for an additional 3 hr. The blue MTT formazan thus formed was dissolved in DMSO, and the absorbance values were measured at 560 nm in a UV-VIS spectrophotometer (Shimadzu UV-2540).

*2.7. Cell Cycle Analysis by Flow Cytometry.* To estimate the percentage of cells at different stages of cell cycle, cultured cells ($3 \times 10^6$), after specified treatment with AECS/p-BQ for 1 hr, were washed with PBS solution and incubated in fresh culture medium for 12 hr. Cell cycle analysis was performed by propidium iodide (PI) according to manufacturer's protocol and analyzed using the FACS Calibur-Cell Quest software. A total of 10,000 events were acquired, and a histogram plot of FL2-H was recorded.

*2.8. Assessment of Apoptosis by Flow Cytometry.* To estimate the percentage of cells undergoing apoptosis, cultured cells ($3 \times 10^6$), after specified treatment with AECS for 1 hr, were washed with PBS solution (Hyclone, Thermo Scientific) and incubated in fresh culture medium for 12 hr. Apoptosis was assessed by Annexin V and propidium iodide (PI) (Becton Dickinson) according to manufacturer's protocol and analyzed using the FACS Calibur-Cell Quest software

(Becton Dickinson) as described earlier [15]. A total of 10,000 events were acquired, and dual parameter dot plot of FL2-H ($x$-axis; PI-fluorescence, linear scale) versus FL1-H ($y$-axis; Annexin V-FITC-fluorescence, linear scale) was recorded.

*2.9. Lysate Preparation, Immunoprecipitation, and Immunoblotting.* Lysate preparation and protein immunoprecipitation were performed as described by Bao et al. [23]. After treatment, cells were extracted in solubilization buffer containing 50 mM Tris, pH 7.5; 150 mM NaCl; 10% glycerol; 1% Nonidet P-40; 1 mM EDTA; Protease Inhibitor Cocktail (Sigma); Phosphatase Inhibitor Cocktail (Sigma). Lysates were cleared by centrifugation at 20,000 g for 10 min at 4°C, and the total protein concentration was estimated. Protein (400 $\mu$g) in the supernatant was immunoprecipitated by overnight incubation with 4 $\mu$g anti-EGFR antibody (Cell Signaling Technology, USA) at 4°C, followed by Protein A-Sepharose CL-42 (GE Healthcare) precipitation for 3 hr at 4°C. Immunoprecipitates were washed 3 times with HNTG buffer containing 20 mM HEPES, pH 7.5; 150 mM NaCl; 0.1% Triton X-100; 10% glycerol, resolved by SDS-PAGE, and transferred to PVDF membrane. Membranes were blocked for 1 hr in Tris-buffered saline, pH 7.5; containing 0.5% Tween 20 and 5% nonfat milk (Bio-Rad), incubated overnight at 4°C with primary antibody (1 : 1000), followed by 1 hr incubation at room temperature with 1 : 3000 dilution of HRP-conjugated secondary antibody (Cell Signaling Technology, USA). Immunoreactive protein bands were detected by chemiluminescence. Blotting antibodies used were anti-EGFR, anti-p-BQ, anti-p53, anti-phospho-p53, anti-Caspase 3, anticleaved Caspase 3, anti-Akt, anti-phospho-Akt, anti-phosphotyrosine-845, anti-phosphotyrosine-1045, anti-phosphotyrosine-1068, anti-phosphotyrosine-1086, anti-ERK1/2, anti-phospho-ERK1/2, anti-c-Fos, anti-c-Cbl, antiubiquitin(UbC3) (Cell Signaling Technology, USA), antiphosphotyrosine PY20, anti-phosphotyrosine-1173, anti-$\beta$ actin (Santa Cruz Biotechnology, USA), anti-HRAS + KRAS, anti-c-Myc, and anti-phospho-c-Myc (phospho T58+S62) (abcam, UK).

*2.10. Detection of Reactive Oxygen Species (ROS) Production.* Prior to treatment, cells were incubated for 30 min with 10 $\mu$M 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) (Sigma-Aldrich). Fluorescent images were captured using confocal laser scanning microscope (LSM 510 META, Carl Zeiss).

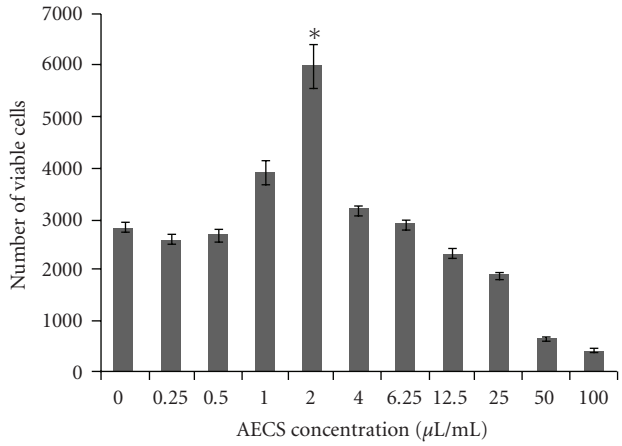
*2.11. Cell Proliferation Assay.* Cell proliferation was assessed using the "In Situ Cell Proliferation Kit, FLUOS" (Roche Applied Science, Germany), according to the manufacturer's protocol. Briefly, after treatment the cells were incubated in serum-free medium containing 10 $\mu$M BrdU Labeling Solution for 5 hr at 37°C. Cells were washed with PBS and fixed for 30 min at 4°C in fixative solution, containing 50 mM glycine (pH 2.0) in 70% (V/V) ethanol. Cells were then permeabilized for 20 min at room temperature with denaturation solution, containing 4 M HCl, followed by subsequent PBS washes until the pH reached above 6.5. To block nonspecific binding, cells were then incubated for 10 min at room temperature in incubation buffer, containing PBS, 0.5% BSA, and 0.1% Tween 20. Cells were then incubated with anti-BrdU-FLUOS antibody for 45 min at 37°C in a humid chamber. After incubation, cells were washed with PBS and analyzed by flow cytometry using the FACS Calibur-Cell Quest software (Becton Dickinson). A total of 10,000 events were acquired, and a histogram plot of FL1-H was recorded.

*2.12. Statistical Analysis.* All values are expressed as mean ± SD. Statistical significance was carried out using one-way ANOVA. The $P$ values were calculated using appropriate $F$-tests. Difference with $P$ values <.05 was considered significant.

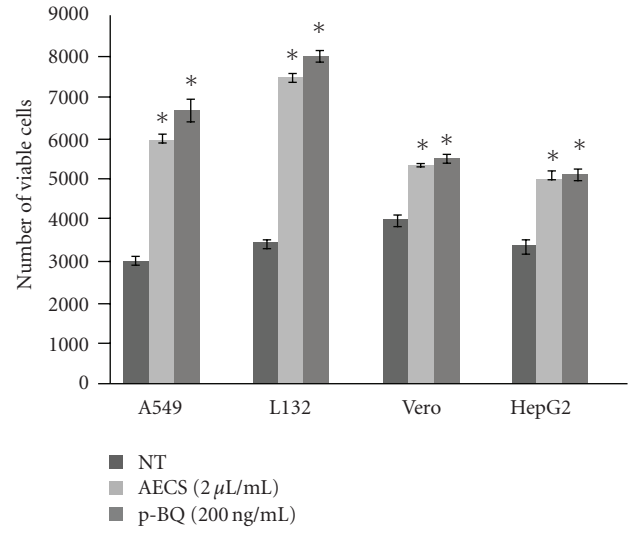
## 3. Results and Discussion

*3.1. Proliferation of Human Lung Epithelial Cells (A549) by AECS/p-BQ.* Using MTT assay here we show that whereas low concentration of aqueous extract of cigarette smoke (AECS) induces proliferation of human lung epithelial cells in culture (A549), high concentrations lead to cell death (Figure 1(a)). The optimum AECS concentration that causes maximum cell proliferation is about 2 $\mu$L/mL. The proliferation is not restricted to A549 cells; it also occurs in other cell lines, such as L132 (normal human lung epithelial cells), Vero (African green monkey kidney cell line), and HepG2 (human liver cell line) (Figure 1(b)). The AECS-induced proliferation is mimicked by the amount of p-BQ (200 ng/mL) produced in the culture medium from 2 $\mu$L/mL of AECS (Figure 1(c)). The amount of p-BQ formed from AECS in the incubation mixture was determined by HPLC. Like that observed with AECS, high concentrations of p-BQ cause cell death (Figure 1(c)). A single treatment with AECS (2 $\mu$L/mL) or p-BQ (200 ng/mL) results in continued proliferation for 24–72 hr (Figure 1(d)).The proliferation by either AECS or p-BQ is completely prevented by antibody to p-BQ (Figure 1(e)). The inhibitory role of anti-p-BQ antibody on the proliferation of A549 cells has also been confirmed by the incorporation of BrdU using flow cytometry analysis (Figures 1(f) and 1(g)). The results indicate that p-BQ derived from AECS is responsible for AECS-induced proliferation of the lung cells. The AECS used was prepared from Kentucky research cigarettes (3R4F). Similar results were obtained by AECS prepared from a commercial cigarette (Wills Navy Cut, India; results not shown), indicating that the observations were not specific to Kentucky research cigarettes.

p-BQ is not present in CS, but is formed from p-benzosemiquinone (p-BSQ), a long-lived semiquinone present in substantial amounts (100–200 $\mu$g/cigarette) in smoke from Kentucky research cigarettes as well as a number of commercial cigarettes examined [15]. p-BSQ is present exclusively in the tar phase of CS and is extracted in the AECS [12, 14]. p-BSQ is converted to p-BQ by disproportionation (2 p-BSQ → p-BQ + HQ) [16], as well as oxidation by




(a)

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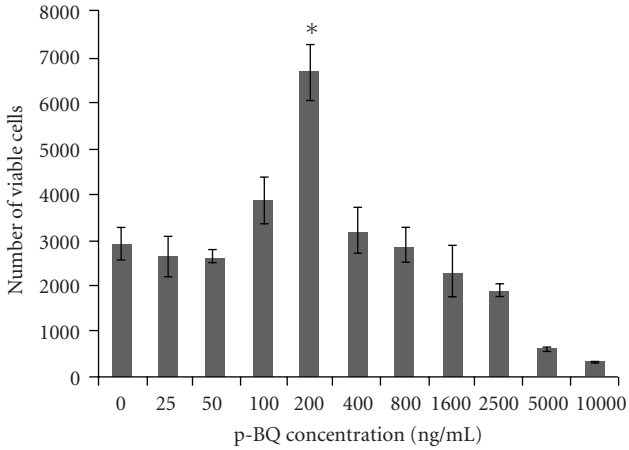


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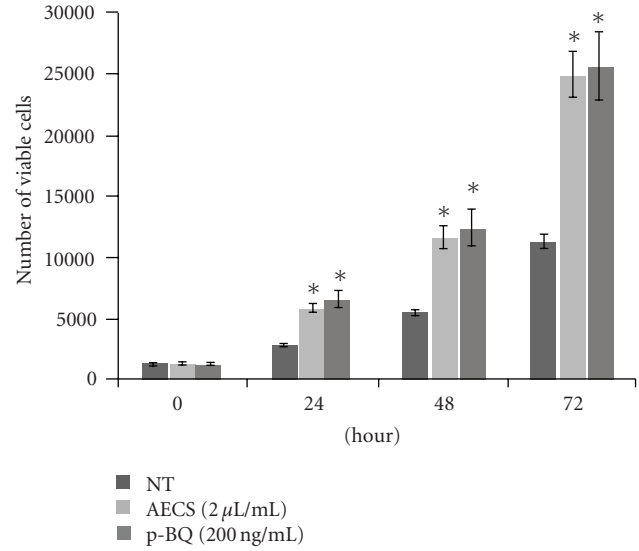
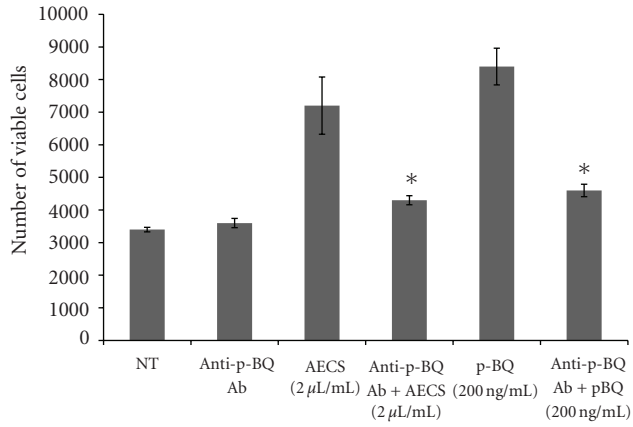
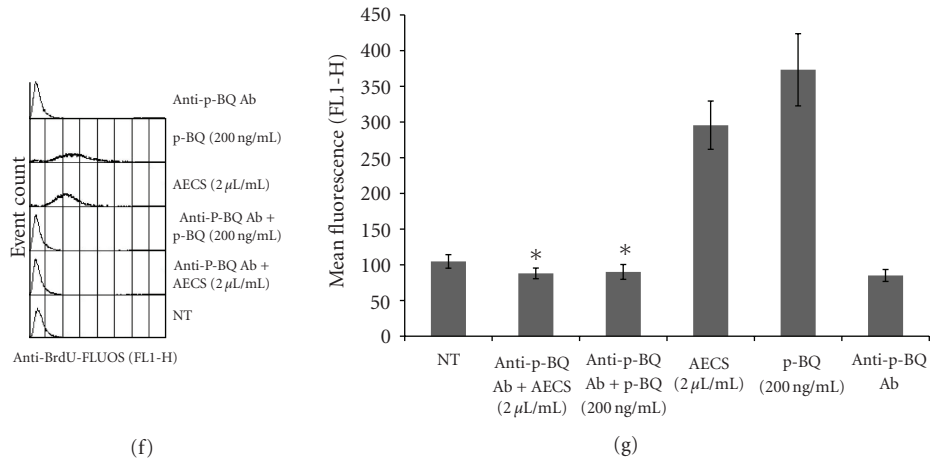



(d)




(e)

Figure 1: Continued.




(f) (g)

Figure 1: AECS/p-BQ-induced proliferation of human lung epithelial cells (A549) and its prevention by anti-p-BQ antibody. Except in (d), all the treatments were made in 24 hr. Effect of concentration gradient of AECS on proliferation and death of A549 cells in culture, as determined by MTT cytotoxicity assay (a). AECS (2 $\mu$L/mL) or p-BQ (200 ng/mL)-induced proliferation in other cell lines, namely. L132, Vero, and HepG2 (b). Effect of concentration gradient of p-BQ on proliferation and death of A549 cells in culture, as determined by MTT cytotoxicity assay (c). Treatment with AECS (2 $\mu$L/mL) or p-BQ (200 ng/mL) results in continued proliferation for 24–72 hr (d). AECS/p-BQ-induced proliferation is prevented by anti-p-BQ antibody, as evidenced by MTT assay (e). The inhibitory role of anti-p-BQ antibody on the proliferation of A549 cells as determined by the incorporation of BrdU using flow cytometry assay (f, g). All data are depicted as the mean ± SD for three independent experiments ($*$ indicates significant difference, $P < .05$ in comparison to nontreated control (a, b, c, d); and in comparison to AECS and p-BQ, resp. (e, g)).

transition metal ($Fe^{3+}$, $Cu^{2+}$) containing proteins (p-BSQ → p-BQ) [14]. The method of preparation of AECS was so devised as to simulate the manner in which the respiratory tract lining fluid is exposed to CS during the process of smoking by humans [18]. Cigarette tar is continually being deposited in the lungs of smokers, and these lungs are continually bathed in an aqueous solution that can solubilize and transport the water soluble components of the tar [12]. p-BSQ present in cigarette tar can be extracted into aqueous solutions and thus would be in solutions bathing a smoker's lung [12]. In CS solution produced in the lungs, p-BSQ would be converted to p-BQ and induce proliferation of cells. This is supported by the observation that CS causes cell proliferation in the lungs of rat in vivo [8].

*3.2. Cell Cycle Analysis of the Proliferating Cells.* The rate of cell proliferation within any population of cells depends on three parameters: (a) the rate of cell division, (b) the fraction of cells within the population undergoing cell division, and (c) the rate of cell loss from the population due to terminal differentiation or cell death. Failure to regulate these functions properly results in an altered phenotype and cancer [19]. Here, we have performed the cell cycle analyses using propidium-iodide (PI) staining followed by flow cytometry. Figure 2(a) shows the histogram plot of A549 cells either nontreated (NT) or exposed to 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 24 hr. The mean fluorescence of 2 $\mu$L/mL AECS or 200 ng/mL p-BQ-treated cells was significantly higher ($P < .05$) than that of the nontreated cells. However, pretreatment with 40 $\mu$g/mL vitamin C for 15 min completely reduces the mean fluorescence (Figure 2(b)). In addition to the relative cellular DNA content, the cell distribution during the various phases of the cell cycle was also determined (Figure 2(c)). Three distinct phases were recognized in the AECS/p-BQ-induced proliferating cell population: the G0-G1 (Region "M1" in Figure 2(a)), S or the DNA synthesis phase (Region "M2" in Figure 2(a)), and the G2-M phase (Region "M3" in Figure 2(a)). Also, the percentages of cells occupying the different phases of the cell cycle were calculated (Figure 2(c)). Figure 2(c) shows that compared to the nontreated cells (30.2%), cells treated with 2 $\mu$L/mL AECS or 200 ng/mL p-BQ have higher number of cells (40.55% or 47.45% of the total cell population, resp.) in the S-phase, indicating markedly higher rate of DNA synthesis. However, pretreatment with 40 $\mu$g/mL vitamin C for 15 min prior to treatment with 2 $\mu$L/mL AECS or 200 ng/mL p-BQ reduces the percentage of cells in S-phase to 30.15% and 31.35%, respectively (Figures 2(a), 2(b), and 2(c)), indicating prevention of higher rate of DNA synthesis.

*3.3. Vitamin C Prevents AECS/p-BQ-Induced Proliferation of Cells.* We have previously shown that CS produces toxicity and tissue damage only in marginal vitamin C-deficient guinea pigs, but not in vitamin C-sufficient ones [14, 18, 20]. We had also shown that a moderately large dose of vitamin C prevents CS-induced toxicity, apparently by reducing and inactivating p-BQ (14). This is because vitamin C ($E^\circ$ = +0.08 V) strongly reduces p-BQ ($E^\circ$ = +0.71 V) to less toxic hydroquinone and thereby inactivates p-BQ. Here, we show that p-BQ derived from AECS causes proliferation of lung cells in serum-free medium (Figure 1), which is essentially free of vitamin C. MTT assay also indicates that 40 $\mu$g/mL vitamin C prevents AECS/p-BQ-induced proliferation of A549 cells (Figure 3(a)). At this concentration, vitamin C

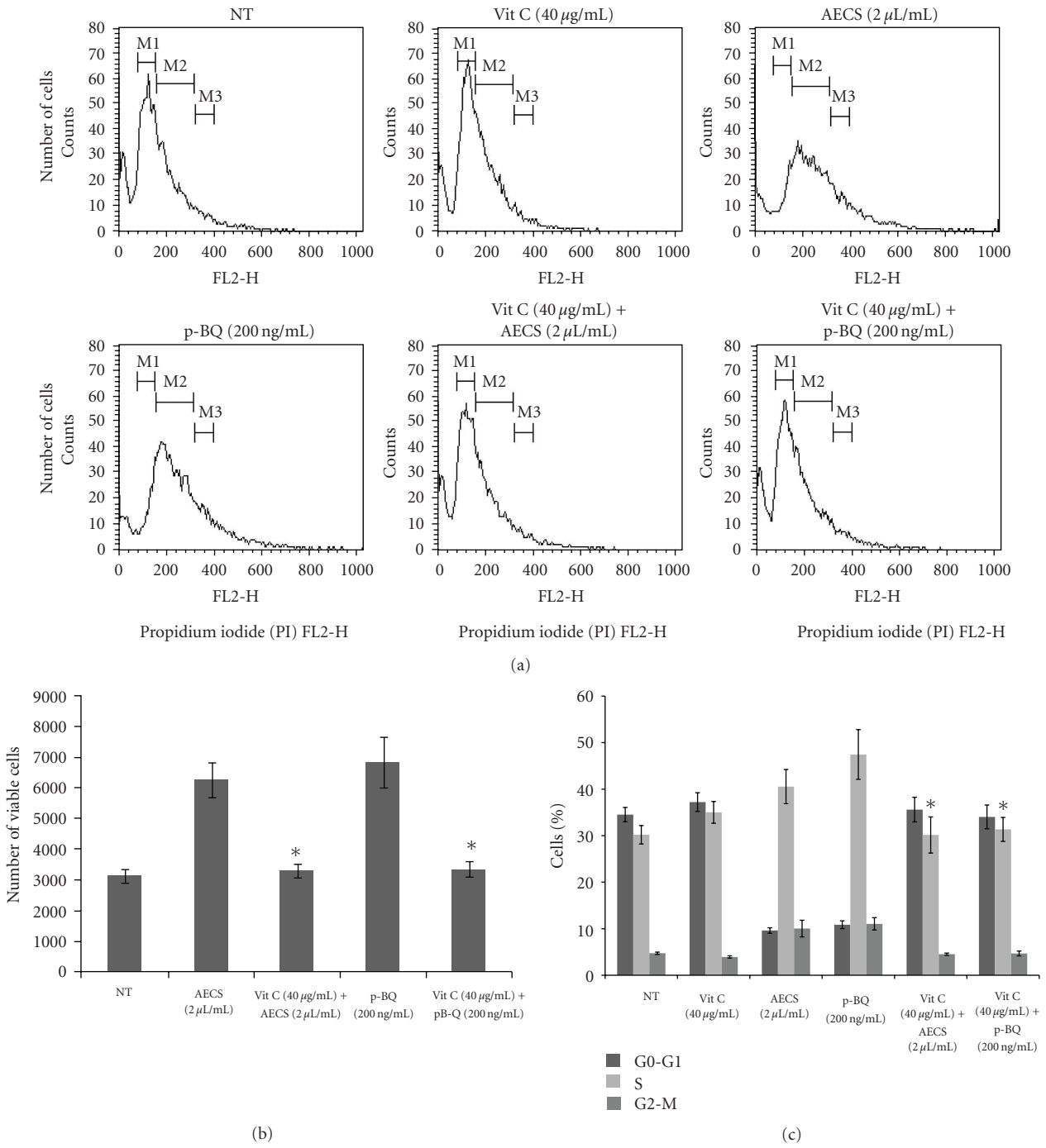



Figure 2: Cell cycle analysis of proliferating A549 cells. Histogram plot (Event count versus FL2-H) of AECS/p-BQ-induced proliferation of A549 cells and its prevention by vitamin C as determined by propidium iodide (PI) staining followed by flow cytometry (a). Bar diagram showing mean fluorescence of PI (FL2-H) in cells either nontreated (NT) or exposed to 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 24 hr and its prevention by vitamin C (40 $\mu$g/mL) (b). Bar diagram showing the relative cell distribution during various phases of the cell cycle after exposure to 2 $\mu$L/mL AECS or 200 ng/mL p-BQ (c). Data are means ± SD for three independent experiments ($*$ in (b) and (c) indicates significant difference, $P < .05$ in comparison to AECS and p-BQ treatment).

does not have any effect on the growth of nontreated (NT) cells in the absence of AECS/p-BQ (data not shown). The inhibitory role of vitamin C has been confirmed by BrdU incorporation, as evidenced by flow cytometry assay (Figures 3(b) and 3(c)).

The role of vitamin C in the prevention and treatment of cancer has a long and controversial history. Although there has been a paucity of human studies using vitamin C to treat already existing cancer, there is considerable epidemiological evidence pointing to the benefits of vitamin C in

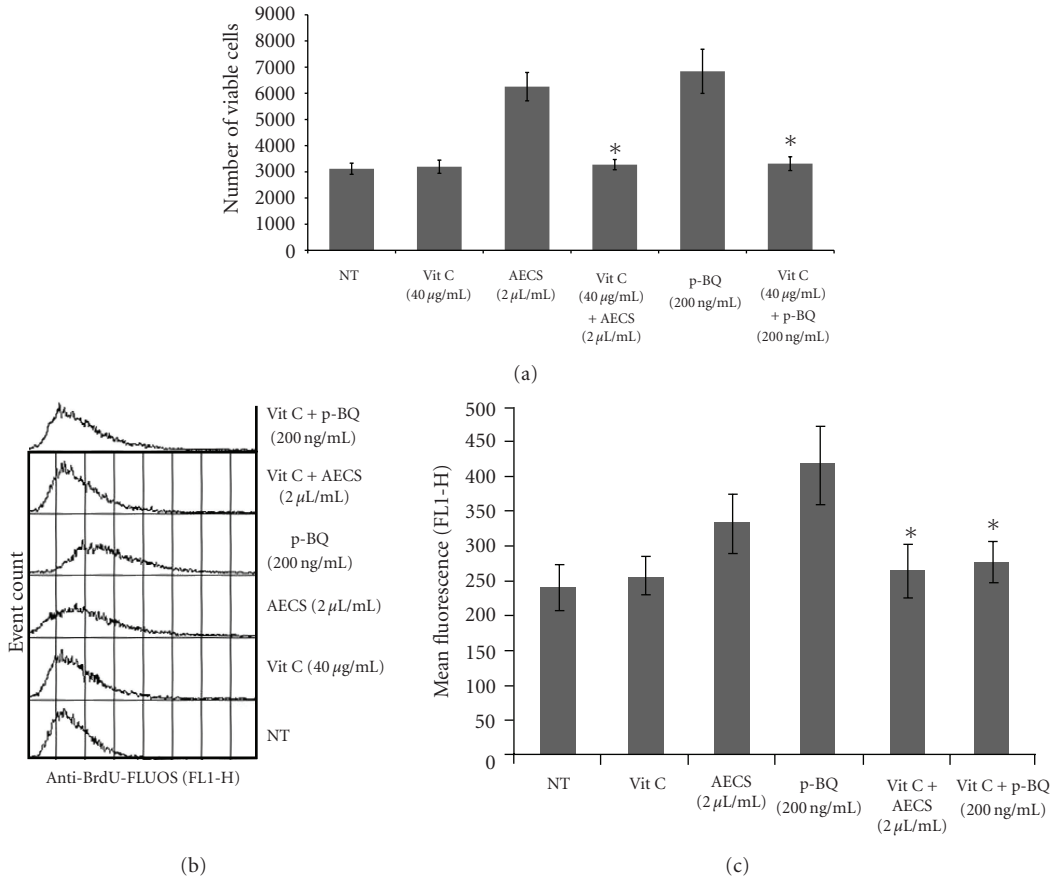



Figure 3: Vitamin C prevents AECS/p-BQ-induced proliferation of cells. AECS/p-BQ-induced proliferation is prevented by vitamin C (40 $\mu$g/mL), as evidenced by MTT assay (a), as well as BrdU-incorporation assay (b and c). Serum-starved A549 cells (2000 cells/well) grown on 96-well tissue culture plates were either nontreated (NT) or exposed to 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 24 hr with or without vitamin C (40 $\mu$g/mL) pretreatment in serum-free medium for 15 min (a, b, and c). Data represent means ± SD for three independent experiments ($*$ indicates significant difference, $P < .05$ in comparison to AECS (2 $\mu$L/mL) and p-BQ (200 ng/mL), resp.).

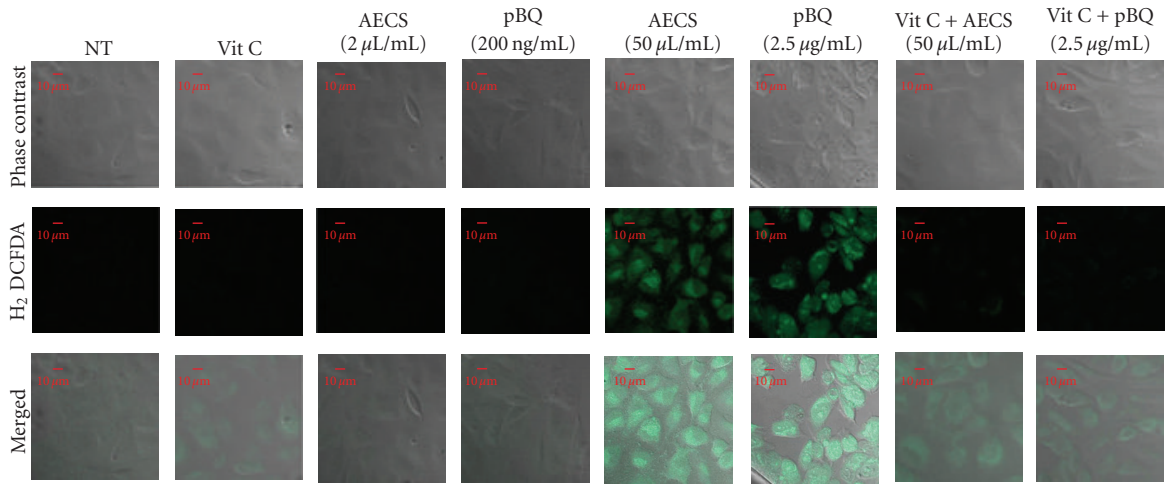



Figure 4: Effect of low or high concentration of AECS/p-BQ on reactive oxygen species (ROS) production in cultured A549 cells and its prevention by vit C. Serum-starved A549 cells grown on coverslips were either nontreated (NT) or exposed to 2 $\mu$L/mL AECS, 50 $\mu$L/mL AECS, 200 ng/mL p-BQ or 2.5 $\mu$g/mL p-BQ for 1 hr with or without vitamin C (40 $\mu$g/mL) pretreatment in serum-free medium for 15 min. After treatment, cells were incubated in fresh media containing $H_2$DCFDA for 30 min and PBS washed twice, and fluorescent images were captured.

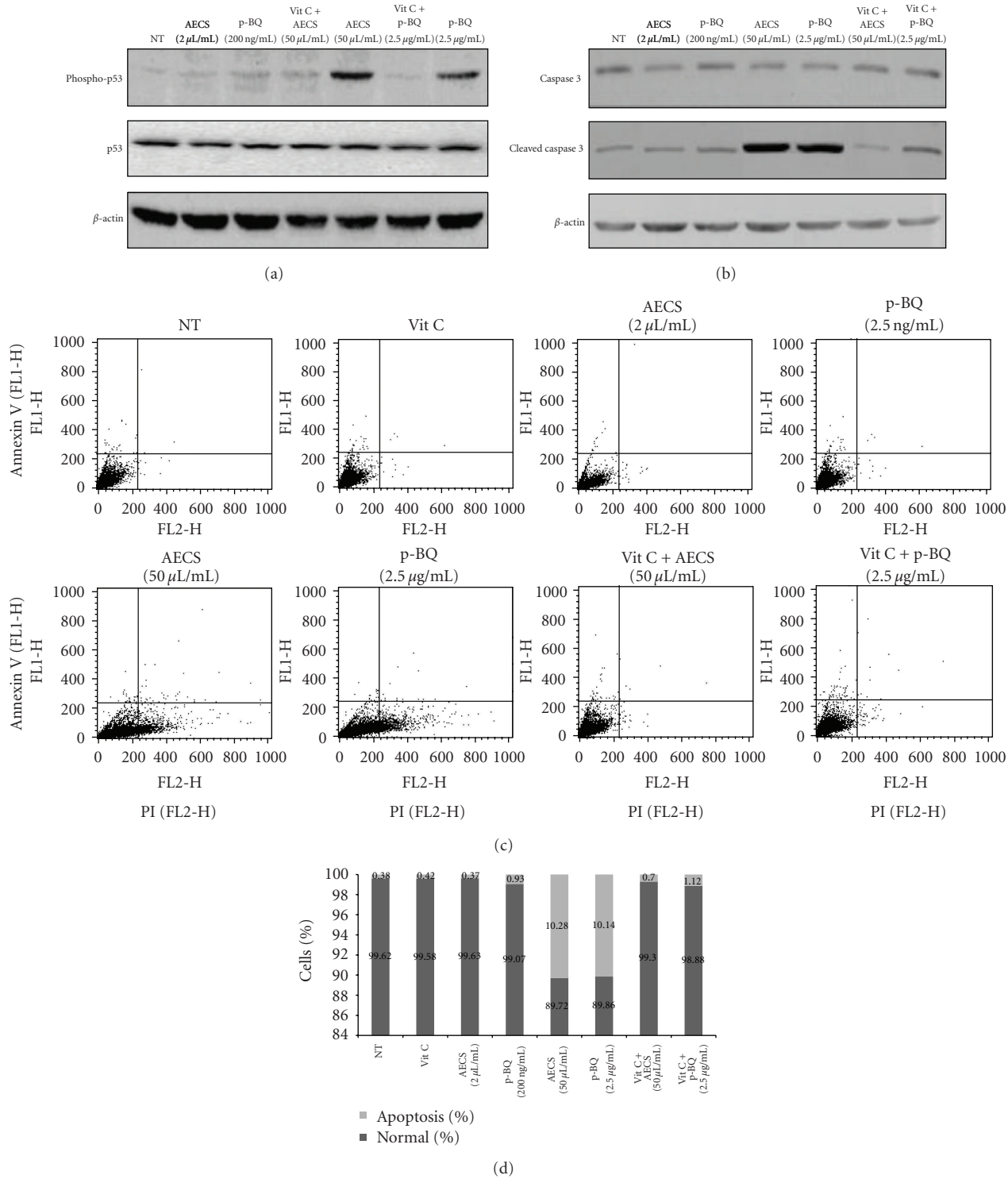



Figure 5: Status of p53, phospho-p53, caspase 3, cleaved-caspase 3 and apoptosis in cultured A549 cells exposed to low or high concentration of AECS/p-BQ. The figure represents immunoblots of phosphorylated p53 and p53 (a) and caspase 3 and cleaved caspase 3 (b). Cell lysate of A549 cells were either nontreated (NT) or exposed to AECS (2 $\mu$L or 50 $\mu$L/mL) or p-BQ (200 ng or 2.5 $\mu$g/mL) for 1 hr followed by incubation in serum containing media for 12 hr. Vitamin C (40 $\mu$g/mL) pretreatment of cells in serum-free media for 15 min prevented AECS/p-BQ-induced activation of p53 or cleavage of caspase 3 (a, b). $\beta$-actin was used as the loading control. Effect of low or high concentrations of AECS/p-BQ on apoptosis in cultured A549 cells and its prevention by vitamin C as evidenced by flow cytometry (c). A549 cells were grown on 60 mm culture plates and were gradually serum starved for 3 days to synchronize the cells. Then the cells were either nontreated (NT) or exposed to 2 $\mu$L/mL or 50 $\mu$L/mL AECS for 1 hr, 200 ng/mL or 2.5 $\mu$g/mL p-BQ for 1 hr, with or without 40 $\mu$g/mL vit C pretreatment in serum-free medium for 15 min. After treatment, cells were incubated in fresh media containing serum for 12 hr, followed by Annexin V-PI assay. Bar graphs show the percentage of normal and apoptotic cells after respective treatments, as evidenced by flow cytometry (d). The numbers within the bars represent percentage of normal and apoptotic cells.

the prevention of a number of types of cancer, including lung cancer [24, 25]. Almost 90% of lung cancer is due to cigarette smoking [2]. We had shown that CS consumes vitamin C [18]. This would corroborate the observations by other researchers that lung cancer patients usually suffer from hypovitaminosis C [25]. Several clinical trials of cancer and vitamin C demonstrated remarkable tolerance and safety for high dose of vitamin C in patients [26].

It has been shown above that in contrast to low concentration, high concentration of AECS/p-BQ (50 $\mu$L/mL AECS or 2.5 $\mu$g/mL p-BQ) results in cell death (Figure 1(a)). The death is apparently caused by oxidative stress and apoptosis. The oxidative stress has been evidenced by the formation of ROS (Figure 4) and apoptosis by the phosphorylation of p53 (Figure 5(a)) and activation (cleavage) of caspase 3 (Figure 5(b)). Apoptosis was supported by Annexin V/PI assay using flow cytometry (Figures 5(c) and 5(d)). Vitamin C prevents cell death apparently by preventing oxidative stress (Figure 4) and apoptosis (Figure 5). No such oxidative stress or apoptosis was observed with low concentration of AECS (2 $\mu$L) or p-BQ (200 ng) that induced proliferation of cells (Figures 4 and 5).

*3.4. Effects of NNK, NNN, and BP on Cell Proliferation.* Cigarette smoke is a complex mixture of 4000 compounds containing carcinogens, including polycyclic aromatic hydrocarbons (PAHs) and nitrosamines. Among PAH, the most extensively studied is benzo[a]pyrene (BP) and among nitrosamines, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) [11]. Compared to p-BSQ (100–200 $\mu$g/cigarette) [15], the concentrations of these carcinogens in smoke from one cigarette are very low: BP, 20–40 ng; NNK, 80–770 ng; NNN, 1.1–2.9 $\mu$g [11, 27]. These carcinogens produce tumor in rodents only at very high doses [11]. Here, we show that under the experimental conditions, NNN, NNK, and BP do not cause any proliferation of A549 cells (Figures 6(a), 6(b), and 6(c)). At high concentrations, the carcinogens are rather inhibitory to the growth of the cells. Moreover, none of them has any synergistic effect on the proliferation of A549 cells by AECS or p-BQ (data not shown). Although BP, NNK, and NNN do not induce proliferation of cells, but they may exert their carcinogenic effects on p-BQ-induced cell proliferation. Proliferation (cell division) triggers mitotic recombination, gene conversion, and nondisjunction. The time interval for DNA repair during mitosis is short. The DNA is also transiently not base paired or bound to histones, therefore making it more sensitive to chance of adduct formation with DNA and mutation by BP, NNK, and NNN, ultimately leading to carcinogenesis.

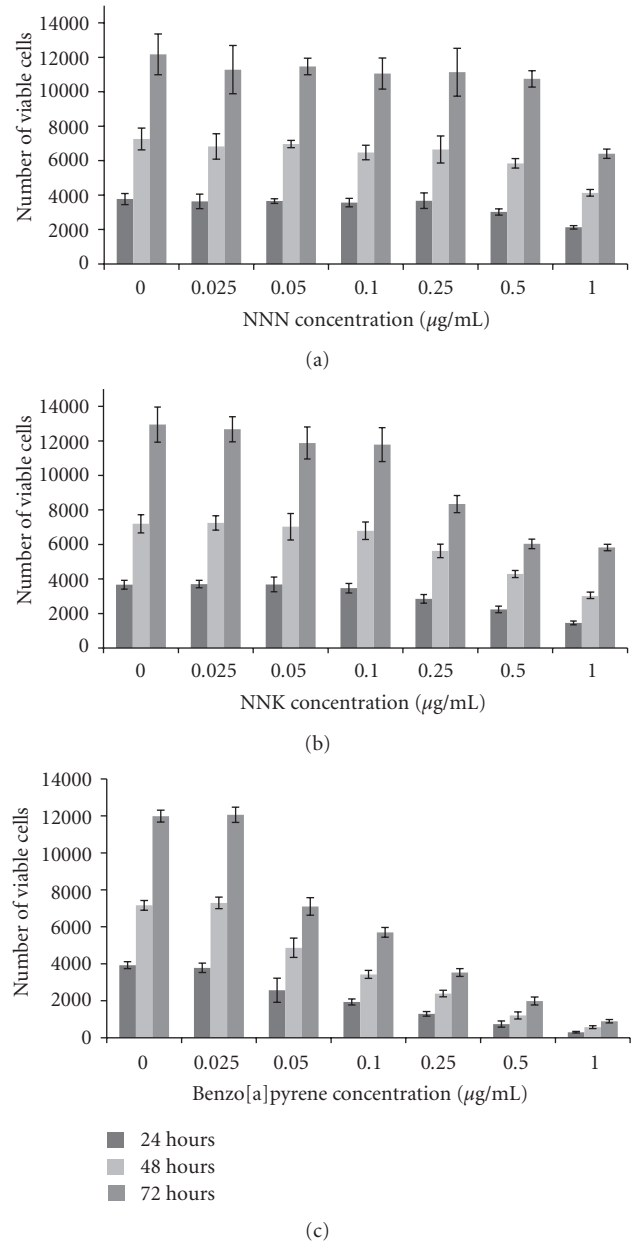



Figure 6: Effects of concentration gradient of NNN (a), NNK (b), and BP (c) on the growth of cultured A549 cells after 24, 48, and 72 hr as evidenced by MTT assay. Serum-starved A549 cells (2000 cells/well) grown on 96-well tissue culture plates were either nontreated (0) or exposed to 0.025, 0.05, 0.1, 0.5, or 1 $\mu$g/mL NNN (a), NNK (b), and BP (c) for 24, 48, and 72 hr. After treatment, MTT cytotoxicity assay was performed. All data are depicted as the means ± SD for four independent experiments.

*3.5. AECS/p-BQ-Induced Cell Proliferation Occurs via the Activation of EGFR That Is Prevented by Vitamin C.* Epidermal growth factor receptor (EGFR) is composed of an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase (receptor tyrosine kinase, RTK)) domain. Activation of the receptor leads to an intracellular signaling cascade that controls cellular proliferation and differentiation. Ligands for these receptors, most importantly epidermal growth factor (EGF) and transforming growth factor-$\alpha$ (TGF-$\alpha$), bind to the extracellular domain resulting in receptor dimerization and autophosphorylation of the intracellular receptor tyrosine kinase (RTK) domain, leading to downstream signaling,

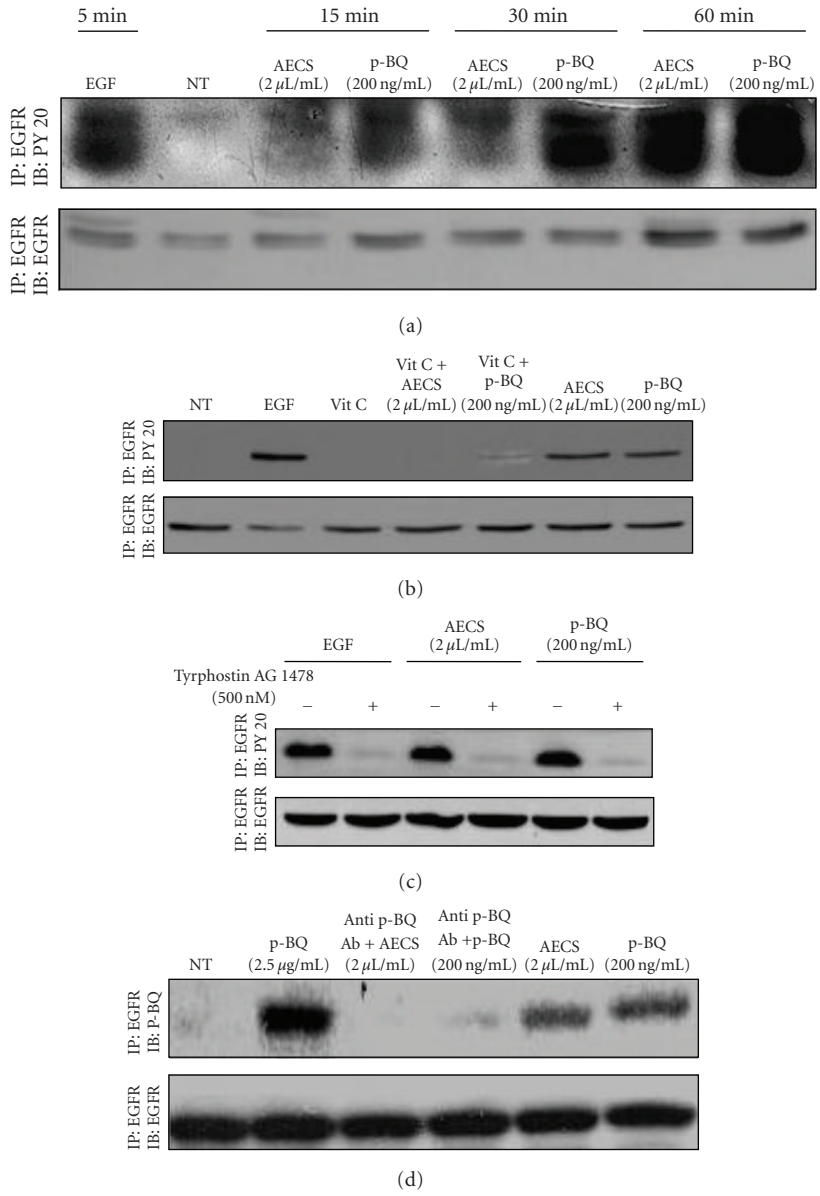



Figure 7: p-BQ mimics AECS in activating EGFR in a time-dependent manner. Serum-starved A549 cells were nontreated (NT) or exposed to 100 ng/mL EGF for 5 min, 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 15 min, 30 min, or 1 hr, respectively (a). Vitamin C (40 $\mu$g/mL) pretreatment of A549 cells for 15 min in serum-free media completely prevented AECS/p-BQ-induced EGFR activation (b). 500 nM Tyrphostin AG1478 (EGFR inhibitor) pretreatment of A549 cells for 15 min in serum-free media completely prevented activation of EGFR by 100 ng/mL EGF, 2 $\mu$L/mL AECS, or 200 ng/mL p-BQ (c). Anti-p-BQ antibody prevented AECS/p-BQ-induced EGFR activation (d). Cells were lysed, and the EGFR was immunoprecipitated (IP) from the cell lysates using anti-EGFR antibody. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted (IB) with either antiphosphotyrosine (PY20) antibody (a, b, c) or with anti-p-BQ antibody (d).

including the activation of *ras*, *raf*, mitogen-activated protein kinase (MAPK), phosphatidyl-3 kinase (PI3K/Akt), and ERK1/2. These molecules are linked to cell growth, proliferation, motility, and survival [28]. Previous studies revealed that EGFR is activated (phosphorylated) in a dose- and time-dependent manner when exposed to CS solution [29]. However, CS is a highly complex mixture, and the component(s) of cigarette smoke solution responsible for cell proliferation has not been known. Using human lung epithelial cells (A549), here we show that p-BQ (200 ng/mL) derived from AECS (2 $\mu$L/mL) is responsible for AECS-induced phosphorylation of EGFR. (Figure 7(a)). The activation (phosphorylation) of EGFR by EGF is mediated by noncovalent interactions, and the phosphorylation appears to be high after 5 min (Figure 7(a)), which decays after 15 min as shown by others [29]. On the other hand, p-BQ derived from AECS covalently binds with the extracellular domain of EGFR tentatively by Michael addition with Lys

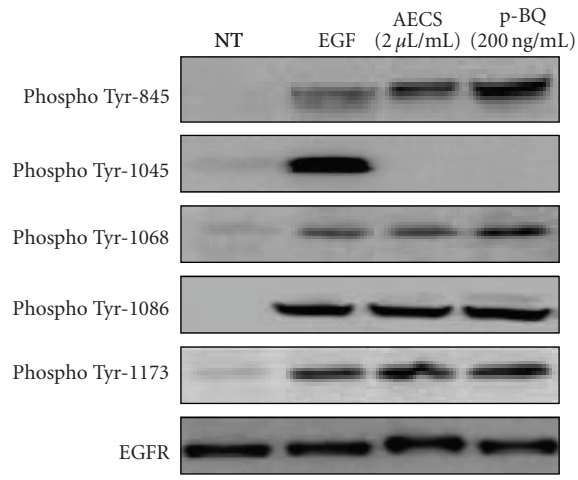



Figure 8: AECS/p-BQ exposure results in aberrant phosphorylation of the EGFR. Serum-starved A549 cells were either nontreated (NT) or exposed to 100 ng/mL EGF for 5 min, 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 1 hr. Cells were lysed, and the EGFR was immunoprecipitated (IP) from the cell lysates using anti-EGFR antibody. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted (IB) with indicated EGFR phosphotyrosine-specific antibodies or anti-EGFR antibodies.

residue [30] and activates the EGFR constitutively. In this case, the phosphorylation by p-BQ starts at about 15 min after treatment and persists for 1 hr, indicating prolonged activation of EGFR. It is reported that constant activation of EGFR results in uncontrolled cell division—a predisposition for cancer, including lung cancer [31, 32]. This would indicate that p-BQ may be a risk factor for the initiation of CS-induced lung cancer. Given that more than 80% of nonsmall cell lung carcinomas (NSCLCs) express EGFR [2], EGFR has become an important therapeutic target for the treatment of these tumors. This led to the development of EGFR inhibitors for anticancer treatment [33, 34]. The most common approaches use either monoclonal antibodies that competitively bind to the extracellular domain or small molecules targeting the intracellular RTK domain. Vitamin C inactivates p-BQ, and we have shown that vitamin C pretreatment of cells prior to AECS/p-BQ exposure completely prevents EGFR activation (Figure 7(b)). This would suggest that intake of vitamin C would prevent lung cell proliferation and initiation of carcinogenesis in smokers. Vitamin C does not prevent EGF-induced phosphorylation of EGFR (data not shown). In order to confirm that AECS/p-BQ induced activation of EGFR, we performed the aforesaid experiment in the presence of 500 nM AG1478 (Tyrphostin, EGFR kinase inhibitor). The results (Figure 7(c)) indicate that 500nM AG1478 completely prevents activation of EGFR by 100 ng/mL EGF, 2 $\mu$L/mL AECS, or 200 ng/mL p-BQ. In a separate coimmunoprecipitation experiment, we show that anti-p-BQ antibody prevents AECS-induced EGFR activation (Figure 7(d)). This would indicate that antibody to p-BQ might also be a candidate for the prevention of the initiation of cell proliferation and lung cancer in smokers.

*3.6. AECS/p-BQ Exposure Results in Aberrant Phosphorylation of the EGFR.* EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity. As a result, autophosphorylation of several tyrosine (Y) residues in the C-terminal domain of EGFR occurs. These include Y845, Y1045, Y1068, Y1148, and Y1173 [35]. This autophosphorylation elicits downstream activation and signaling by several other downstream signaling proteins that initiate several signal transduction cascades, principally the MAPK, Akt, and JNK pathways, leading to DNA synthesis and cell proliferation. Such proteins modulate phenotypes including cell proliferation [36]. Earlier studies indicate that CS exposure resulted in the aberrant phosphorylation of the EGFR [29]. Because p-BQ mimics AECS in activating the EGFR, we wanted to see whether p-BQ also causes aberrant phosphorylation of EGFR. Immunoblot analyses of EGFR from A549 cells exposed to 100 ng/mL EGF for 5 min, 2 $\mu$L/mL AECS for 1 hr, or 200 ng/mL p-BQ for 1 hr show that AECS/p-BQ exposure results in similar aberrant phosphorylation pattern that is distinctly different from EGF exposure (Figure 8). After incubation with AECS/p-BQ, Tyr-845 is hyperphosphorylated, but Tyr-1045 is not phosphorylated. With EGF exposure, Tyr-1045 is strongly phosphorylated, whereas Tyr-845 is phosphorylated to a much lesser extent. The pattern of phosphorylation of Tyr-1068, 1086, and 1173 appears to be almost similar irrespective of treatment with EGF, AECS, and p-BQ.

*3.7. EGFR Exposed to AECS/p-BQ Cannot Bind c-Cbl and Is Not Ubiquitinated.* c-Cbl (120 kDa) is an E3 ubiquitin ligase that plays a crucial role in downregulating the EGFR. On EGFR activation, c-Cbl associates with phosphorylated Tyr-1045 and ubiquitinates the receptor, marking it for clathrin-mediated endocytosis and recognition by the lysosomal machinery, which results in receptor degradation and signal termination [37–40]. EGFR exposed to 100 ng/mL EGF, its cognate ligand, is associated with c-Cbl and ubiquitinated (Figure 9(a)). However, EGFR exposed to 2 $\mu$L/mL AECS for 1 hr or 200 ng/mL p-BQ for 1 hr is not phosphorylated on Tyr-1045, which renders it unable to associate with c-Cbl and precludes it from being ubiquitinated (Figure 9(a)). Therefore, under AECS/p-BQ exposure, c-Cbl loses its ability to bind to EGFR and thereby lacks ubiquitination and degradation, which leads to prolonged signaling even after removal of the external stimuli AECS/p-BQ.

In order to study the fate of EGFR after incubation with AECS/p-BQ followed by removal of these ligands, we continued incubation of the cells for 2 hr more in serum-free medium. The results (Figure 9(b)) indicate that EGFR phosphorylation persists up to 2 hr after pretreatment with 2 $\mu$L/mL AECS or 200 ng/mL p-BQ followed by removal of the ligands. This demonstrates that signals from activated EGFR prolong apparently due to the inability of EGFR degradation after AECS/p-BQ exposure. On the other hand, when the cells were pretreated with 100 ng/mL EGF for 5 min followed by removal of EGF, EGFR phosphorylation does not prolong more than 15 min [29]. Here we show that

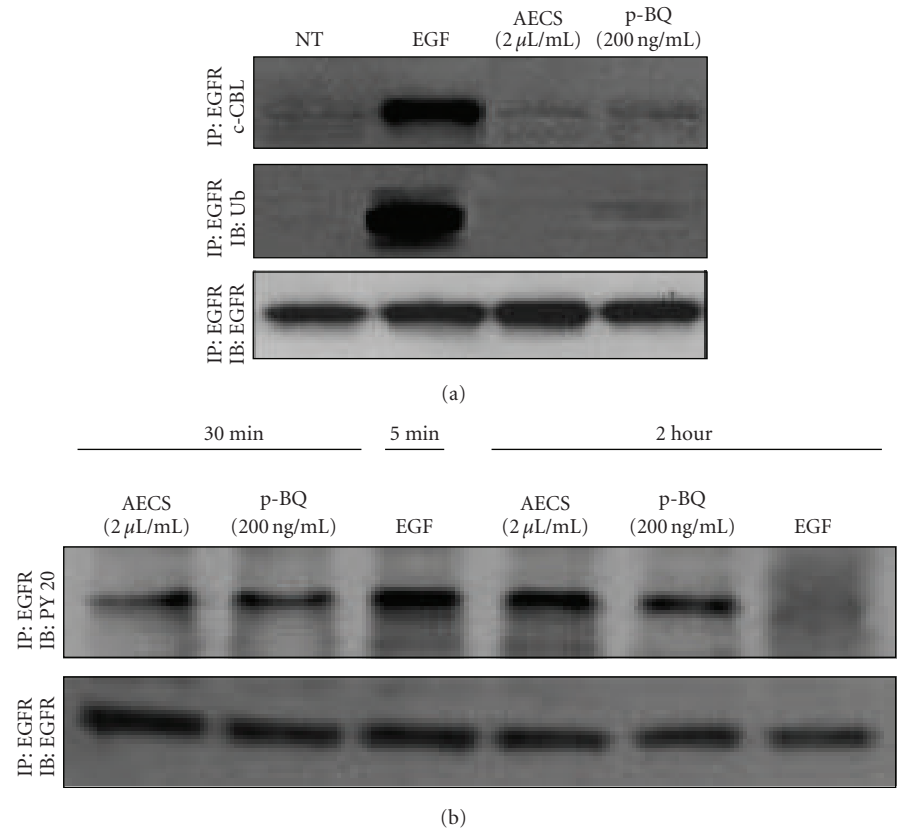



Figure 9: EGFR exposed to AECS/p-BQ cannot bind c-Cbl and is not ubiquitinated. Serum-starved A549 cells were either nontreated (NT) or exposed to 100 ng/mL EGF for 5 min (used as positive control), 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 1 hr. After treatment, cells were immediately lysed, and the EGFR was immunoprecipitated (IP) from the cell lysates using anti-EGFR antibody. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted (IB) with the indicated antibodies (a). Serum-starved A549 cells were either nontreated (NT) or exposed to 100 ng/mL EGF for 5 min or 2 hr, 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 1 hr. After treatment, cells were washed with PBS and further incubated in fresh serum-free medium for 2 hr at 37°C before lysis. The EGFR was immunoprecipitated (IP) from the cell lysates using anti-EGFR antibody. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted (IB) with antiphosphotyrosine (PY20) antibody (b).

phosphorylation of EGFR by EGF is practically nil after 2 hr (Figure 9(b)).

*3.8. AECS/p-BQ Exposure Activates Hras + Kras Which Leads to Downstream Survival and Proliferative Signaling ERK 1/2 and Akt.* The Ras proteins are GDP/GTP-binding proteins that act as intracellular signal transducers. The inactive forms are GDP bound. They are activated by receptor tyrosine kinases including EGFR. The most well studied members of the *ras* gene family are *Hras* and *Kras*. These genes encode immunologically related proteins with a molecular mass of 21 kDa and are homologs of rodent sarcoma virus genes that have transforming abilities. While these wildtype cellular proteins in humans play a vital role in normal tissue signaling, including proliferation, differentiation, and senescence, mutated or overexpressed genes are potent oncogenes that play a major role in many human cancers including lung cancer. Here, we show that exposure to 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 1 hr results in the overexpression of Hras + Kras proteins (Figure 10, row 1).

In addition to Hras and Kras, two well-established mediators of proliferation and cell survival, extracellular signal-regulated kinase (ERK1/2), also called the mitogen-activated protein kinase (MAPK), and Akt (also known as protein kinase B), are known to be involved in cell transformation when persistently activated [41–43]. It is known that activation (phosphorylation) of the ERK1/2 pathway is involved in malignant transformation both *in vitro* and *in vivo*. It is also reported that activation of ERK 1/2 is associated with nonsmall cell lung cancer (NSCLC), 80% of which is caused by cigarette smoking [44]. ERK 1/2 is activated by dual phosphorylation on both Thr202 and Tyr204 residues. Akt is a serine/threonine protein kinase that plays a key role in multiple cellular processes, including promotion of cell survival in several cell lines. Activation of both ERK 1/2 and Akt in a variety of cells is mediated mainly by growth factor receptors that require EGFR phosphorylation [45]. Furthermore, lack of EGFR turnover has been shown to mediate tumor promotion in nonneoplastic rat liver epithelial cells [46]. Figure 10 (rows 2 and 4) shows that exposure to 2 $\mu$L/mL AECS or 200 ng/mL

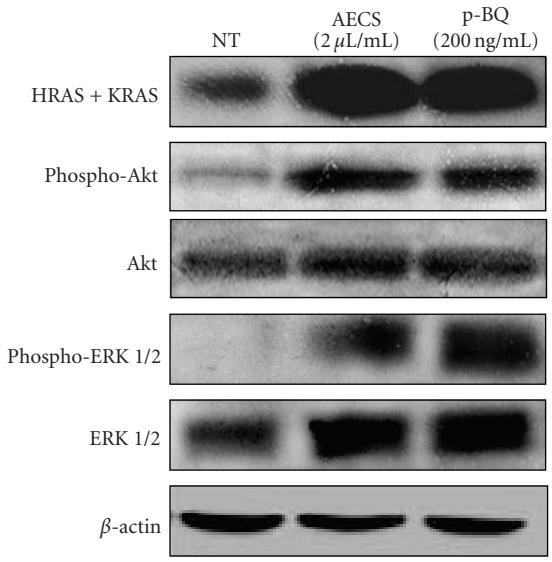



Figure 10: Exposure of A549 cells to AECS/p-BQ results in overexpression of Hras + Kras and activation of downstream proliferation and survival signaling Akt and ERK 1/2. Serum-starved A549 cells were either nontreated (NT) or exposed to 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 1 hr followed by incubation in serum containing media for 6 hr. After 6 hr, cells were lysed, and cell lysates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antiphosphotyrosine (PY20), anti-HRAS + KRAS, anti-phospho-Akt, anti-Akt, anti-phospho-ERK 1/2, and anti-ERK 1/2 antibodies. $\beta$-actin was used as the loading control.

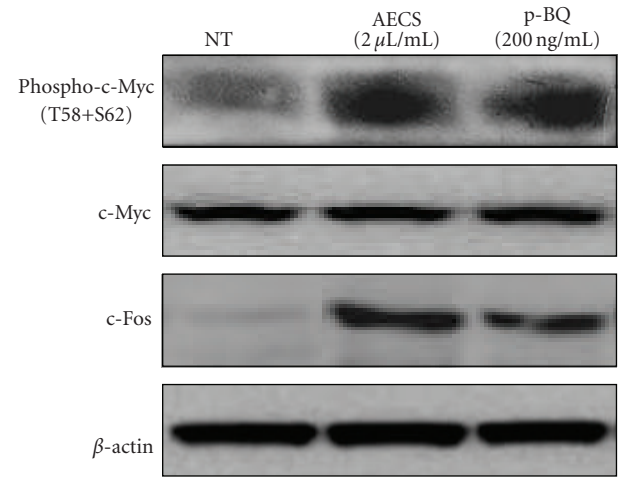



Figure 11: Exposure of A549 cells to AECS/p-BQ results in phosphorylation of c-Myc at Thr 58/Ser 62 and overexpression of c-Fos. Serum-starved A549 cells were either nontreated (NT) or exposed to 2 $\mu$L/mL AECS for 1 hr or 200 ng/mL p-BQ for 1 hr followed by incubation in serum containing media for 6 hr. After 6 hr, cells were lysed, and cell lysates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-phospho-c-Myc (phospho Thr58 + Ser62), anti-c-Myc, and anti-c-Fos antibodies. $\beta$-actin was used as loading control.

p-BQ for 1 hr results in the phosphorylation of downstream ERK 1/2 and Akt signals that render them active. However, the nontreated cells do not show any overexpression of activated Hras + Kras, ERK 1/2, or Akt (Figure 10, rows 3 and 5).

*3.9. AECS/p-BQ Exposure Results in Activation of c-Myc and Overexpression of c-Fos.* The c-Myc protein (49kDa) is a transcription factor, which is encoded by the *c-Myc* gene on human chromosome 8q24. The c-Myc oncoprotein is among the most potent transforming agents in human cells. Elevated levels of the c-Myc oncoprotein contribute to the initiation and progression of most human tumors [47–49]. Increased expression of c-Myc induces proliferation and inhibits differentiation. c-Myc is commonly activated in a variety of tumor cells and can either activate or repress the expression of specific target genes associated with various biological functions. Via this transcriptional regulatory activity, c-Myc contributes to diverse aspects of cancer biology, including cell cycle progression, angiogenesis, metastasis, cell adhesion, cell growth, and genomic instability. Studies revealed a functional association between phosphorylation of c-Myc at Thr58/Ser62 by ERK 1/2 in cell proliferation and cell cycle regulation [50].

*c-Fos* belongs to the Fos family of nuclear oncogenes. The expression of c-Fos protein (62 kDa) is rapidly and transiently induced by a variety of extracellular stimuli, including growth factors. In addition to increased expression, phosphorylation of Fos proteins by ERK kinases in response to external stimuli may further increase transcriptional activity [51–54]. Deregulated expression of c-Fos can result in neoplastic cellular transformation [51]. Figure 11 shows that exposure of A549 cells to 2 $\mu$L/mL AECS or 200 ng/mL p-BQ for 1 hr results in phosphorylation of c-Myc protein at Thr58/Ser62 and overexpression of c-Fos. However, the nontreated cells neither show any c-Myc phosphorylation nor c-Fos expression.

## 4. Conclusion

Despite major advances in the treatment and management of lung cancer, most patients with lung cancer eventually die of this disease. Because conventional therapies have failed to make a major impact on survival, newer approaches are necessary in the battle against lung cancer [55]. It is known that cigarette smoking is the strongest risk factor for developing lung cancer. Eventually the best method to prevent lung cancer is cessation of smoking, which has proven difficult to achieve and unlikely to be accomplished. This has motivated an intense interest in the chemoprevention of this disease [56]. Cigarette smoke (CS) is a complex mixture of about 4000 compounds [11, 12], and identifying the risk factor in CS is essential for achieving this goal. We have identified p-benzoquinone (p-BQ) as a risk factor that is tentatively produced from p-benzosemiquinone [13–15] of aqueous extract of CS (AECS). Lung cancer is believed to arise after a series of progressive pathologic changes (preneoplastic lesions) that are initiated by proliferation

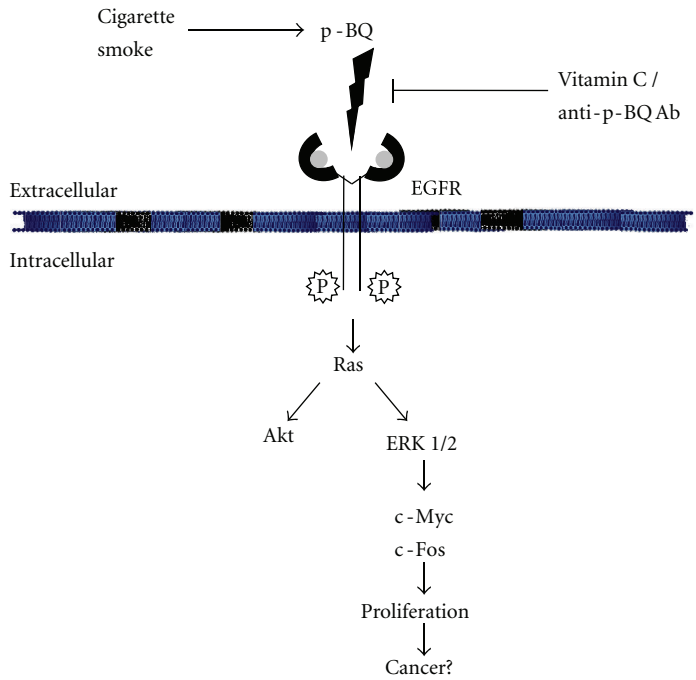



Figure 12: Model showing the molecular mechanisms of cigarette smoke-induced proliferation of human lung epithelial cells (A549) and prevention by vitamin C as well as antibody to p-Benzoquinone.

[4]. We have shown that low concentration of AECS or equivalent amount of p-BQ derived from AECS causes excessive proliferation of human lung epithelial cells (A549) that is mediated via aberrant phosphorylation of EGFR resulting in persistent activation of EGFR. The prolonged activation of EGFR is accompanied by activation of Ras, the downstream survival, and proliferative signaling molecules Akt and ERK1/2, as well as the transcription factors c-Myc and c-Fos. Given that more than 80% of CS-induced nonsmall cell lung carcinomas (NSCLCs) express EGFR [2], inhibition of EGFR has become an important therapeutic target for the treatment of these tumors [28, 57, 58]. We have demonstrated that both anti-p-BQ antibody and vitamin C prevent AECS/p-BQ-induced activation of EGFR and proliferation of lung cells (Figure 12). Vitamin C prevents AECS/p-BQ-induced proliferation apparently by reducing and thereby inactivating p-BQ. We consider that prevention of CS-induced proliferation of lung cells by vitamin C and/or anti-p-BQ antibody may provide a novel intervention for preventing initiation of CS-induced lung cancer.

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*Research Article*

# Short-Term Exposure to Tobacco Toxins Alters Expression of Multiple Proliferation Gene Markers in Primary Human Bronchial Epithelial Cell Cultures

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The biological effects of only a finite number of tobacco toxins have been studied. Here, we describe exposure of cultures of human bronchial epithelial cells to low concentrations of tobacco carcinogens: nickel sulphate, benzo(b)fluoranthene, N-nitrosodiethylamine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). After a 24-hour exposure, *EGFR* was expressed in cell membrane and cytoplasm, *BCL-2* was expressed only in the irregular nuclei of large atypical cells, *MKI67* was expressed in nuclei with no staining in larger cells, cytoplasmic *BIRC5* with stronger nuclear staining was seen in large atypical cells, and nuclear *TP53* was strongly expressed in all cells. After only a 24-hour exposure, cells exhibited atypical nuclear and cytoplasmic features. After a 48-hour exposure, *EGFR* staining was localized to the nucleus, *BCL-2* was slightly decreased in intensity, *BIRC5* was localized to the cytoplasm, and *TP53* staining was increased in small and large cells. *BCL2L1* was expressed in both the cytoplasm and nuclei of cells at 24- and 48-hour exposures. We illustrate that short-termexposure of a bronchial epithelial cell line to smoking-equivalent concentrations of tobacco carcinogens alters the expression of key proliferation regulatory genes, *EGFR, BCL-2, BCL2L1, BIRC5, TP53*, and *MKI67*, similar to that reported in biopsy specimens of pulmonary epithelium described to be preneoplastic lesions.

## 1. Background

Cigarette smoking is a major cause of lung cancer. 90% of male and 75–80% of female lung cancer deaths in the USA are smoking related [1]. As defined by the International Agency for Research on Cancer (IARC), each cigarette contains a mixture of more than 60 known carcinogens [2]. At least twenty of these carcinogens have been linked to tumors [1].

Bronchial epithelium undergoes a stepwise preneoplastic process encompassing various morphological and molecular changes before overt development of lung cancer [3]. The 5-year survival for patients with lung cancer is approximately 15% [4], and patients with nonsmall cell lung cancer in stage I-A disease have a 33% chance of recurrence within 5 years after complete surgical resection [5, 6]. Currently, there is no immunohistochemical or morphological marker, available for metaplasia, dysplasia, or carcinoma insitu, which reliably predicts the biological behavior of preneoplastic lesions.

The *BCL-2* [7–9] and *BCL2L1* antiapoptotic genes are expected to contribute to carcinogenesis. *BCL-2* prolongs survival of noncycling cells and inhibits apoptosis of cycling

cells [10, 11]. Epidermal growth factor receptor (*EGFR*) is a tyrosine kinase receptor which increased cell proliferation [12–16]. *BIRC5* is a member of the inhibitor of apoptosis protein (IAP) family, a cell-cycle-regulated bifunctional protein expressed in G2/M phase [17–19]. *BIRC5* may overcome G2/M phase checkpoints to enforce progression of cells through mitosis, favoring development of neoplastic clones. *MKI67* is expressed during all active phases of the cell cycle. The fraction of *MKI67*-positive tumor cells (*MKI67* labeling index) provides correlation with the clinical course of disease [20–22].

While malignant transformation can be induced in bronchial epithelial cell cultures, the effects of exposure to individual tobacco carcinogens have not been well studied during phases preceding the development of overt cancer. The purpose of our current work is to study the effect of individual tobacco carcinogens on cultured human bronchial epithelial cells.

## 2. Materials and Methods

*2.1. Reagents.* Sources of the toxins, cell line, antibodies, and reagents used in this work are listed in supplementary data (see the Supplementary Material available online at doi:10.1155/2011/208563).

*2.2. Cell Culture.* Bronchial epithelial growth medium (BEGM) was prepared as previously described [23]. The cryopreserved cell line was thawed and initially grown in 35 mm plastic dishes in the above-specified medium in a humidified 5% $CO_2$ incubator at 37°C until cells were 70–80% confluent. Cells were lifted by trypsinization and replated in 24-well plates containing glass cover slips until they are 70–80% confluent.

*2.3. Toxin Exposure.* Toxin solutions (Table 1) were evaluated for effects on nonspecific esterase (NSE) and cytomorphology assessed by PAP staining and phase contrast microscopy. We evaluated the effects of nickel sulphate (heavy metal), benzo(b)fluoranthene (polyaromatic hydrocarbon), N-nitrosodiethylamine (a tobacco nitrosamine), and NNK (a nicotine derivative) using electron microscopy and immunohistochemistry after 24 and 48 hours of exposure at low carcinogen concentration. The final concentration of solvents in the culture media was less than 0.01% as previously described [24]. The working concentration of toxins were based on the epithelial exposure to toxin typically present in one cigarette [25]. The median concentration of each toxin in one smoked cigarette was taken as the medium concentration (M), and lower (L) and higher (H) dose exposure concentrations were arbitrarily determined (Table 1). Two controls were included with each carcinogen exposure, a "solvent control" (S) corresponding to the solvent used to dissolve the toxin (used equivalent to the highest concentration) and a "negative control" (N) containing only the growth medium. Cells were incubated in the culture media containing toxins or controls for 24 and 48 hours. Cells were then washed with DPBS. For Pap staining, cells were fixed in 95% alcohol for 30 minutes, air-dried, and stored at 4°C. For electron microscopy, cells were trypsinized, washed twice in growth medium and, centrifuged. The cell pellet was fixed in 2.5% glutaraldehyde and refrigerated at 4°C.

*2.4. Nonspecific Esterase (NSE) Cytochemistry and Papanicolaou (PAP) Staining.* We used NSE staining as a measure of cell activity and to determine the minimally toxic concentration of tested chemicals that is capable of inducing a meaningful change in the cytomorphology. The staining was done as previously described [26, 27]. The stained cells on the cover slips were air-dried and mounted inverted on glass slides using Permount mounting media (Fisher Scientific, Pittsburgh, PA).

*2.5. Immunohistochemistry (IHC).* Fixed cytospin slides were incubated in 3% hydrogen peroxide for 15 minutes at room temperature, rinsed in distilled water, incubated for 10–15 minutes at room temperature with blocking serum, followed by the application of primary mouse monoclonal antibodies for *EGFR* (1 : 100), *TP53* (1 : 100), *BIRC5* (1 : 150), *MKI67* (1 : 50), *BCL-2* (1 : 100), and *BCL2L1* (1 : 150). The primary antibodies were incubated for 60 minutes at room temperature. The slides were washed three times with PBS 0.2% Tween followed by application of biotin-labeled antimouse IgG and further incubated for 30 minutes at room temperature. The cells were then washed with PBS 0.2% Tween, and a working dilution of fresh DAB solution was added. Slides were counterstained in fresh Gill's hematoxylin, placed in ammonia water for 5–10 seconds, dehydrated, and mounted with Permount.

*2.6. Electron Microscopy (EM).* Standard tissue processing for electron microscopy was used [28]. Toxin-treated glutaraldehyde-fixed cell pellets ware washed in Millonig's phosphate buffer and placed in 1% osmium tetroxide for one hour, washed twice with Millonigs buffer, dehydrated by passing through graded ethanol twice, 15 minutes each, and finally left in 100% ethanol for 30 minutes. Then, the pellet was treated with 1 : 1 and 3 : 1 working Spurr : ethanol mixtures followed by a 100% working Spurr solution, 30 minutes each, and embedded in the center of a beam capsule. Thick and thin sections were prepared and examined with a transmission electron microscope (Tecnai 12 T; FEI Company, Hillsboro, Oregon).

*2.7. Phase Contrast Microscopy.* All slides after defined exposure times and prior to any further processing were viewed using an inverted phase contrast microscope (Leica DMIRE2, Germany) at 20x, and appropriate images were taken. Regular digital images of stained slides were taken at 60x for morphometry using a microscope (Olympus BX51, Center Valley, PA) fitted with a digital camera (Olympus DP71, Center Valley, PA). A bar of 100 microns was drawn using an internal scale at 60x, later used to calibrate ImageJ software.

*2.8. Evaluation of NSE, ki-67, p53 Staining, and Morphometry.* NSE staining was evaluated in 100 cells under 60x objective. Negative cells were graded as zero, minimal

Table 1: Tobacco carcinogens and dilutions used.

| No. | Tobacco carcinogens | Toxin range: ng/cigarette | Working concentrations | | | Solvent used for stock soln. |
|---|---|---|---|---|---|---|
| | | | High ng/ml | Med[#]ng/ml | Lowng/ml | |
| (1) | Nickel Sulphate** | 0–510 | 800 | 500 | 200 | Water |
| (2) | Cadmium Chloride | 0–6670 | 1200 | 700 | 200 | Water |
| (3) | Chromium Chloride | 0.2–500 | 800 | 500 | 200 | Water |
| (4) | Sodium Selenite | 0–1400 | 1900 | 1400 | 900 | Water |
| (5) | Benzo(b)fluoranthene** | 4–22 | 44 | 22 | 2 | Acetone |
| (6) | Indeno(1,2,3,-d)pyrene | 4–20 | 40 | 20 | 2 | ETOH |
| (7) | Ethyl Carbamate | 20–38 | 80 | 40 | 2 | ETOH |
| (8) | Dibenz(a,h)anthracene* | 4 | 12 | 4 | 1 | Toluene |
| (9) | N-Nitrosodiethylamine** | 0–2.8 | 9 | 3 | 1 | ETOH |
| (10) | 5-Methylchrysene | 0.6 | 3 | 1 | 0.25 | ETOH |
| (11) | Dibenzopyrene | 1.7–3.2 | 9 | 3 | 1 | ETOH |
| (12) | Dibenz(a,h)acridine | 0.1 | 3 | 0.1 | 0.25 | Acetone |
| (13) | NNK** | 80–770 | 1300 | 800 | 300 | Water |
| (14) | Benzo(k)fluoranthene* | 6–12 | 24 | 12 | 2 | Toluene |

[*] Excluded from morphometry and immunohistochemical analysis due to cytotoxicity.
[**] Used in immunohistochemistry only.
[#] Equivalent volume of solvent/mL BEGM used as solvent (S) control in NSE staining only.

staining and/or forming an incomplete rim around the nucleus whether punctate or homogeneous as 1, moderate staining and/or forming a complete rim around the nucleus whether punctate or homogeneous as 2, and strong staining forming a complete rim around the nucleus as 3. Some small pyknotic nonstaining cells, most probably representing basal cells, identified both in the unexposed and exposed cultures were not counted. For morphometry of cytological effects, nuclear:cytoplasmic ratios were determined with NIH ImageJ software. Manual threshold of an 8-bit greyscale image was performed and regions of interest were selected with the "region of interest" (ROI) manager. A line-drawing tool was used to select areas not amenable to thresholding. ImageJ was calibrated in a set scale window by using the 100 $\mu$m scale bar captured earlier at 60x magnification. For density calibration, ImageJ was calibrated following the procedure listed at the HIN ImageJ manual (http://rsb.info.nih.gov/ij/). Biostatistical analysis and graphical displays were done using R software (http://www.r-project.org/).

## 3. Results

*3.1. NSE, PAP Staining, and Morphometry.* The morphology of untreated cells was used as the basis for comparison. These cells displayed regular and smooth cellular and nuclear contours and small nucleoli. All carcinogen-treated cells displayed a range of morphologic changes; a representative image depicting benzo(b)fluoranthene-treated cells is displayed in Figure 1 (top panel). At low concentration, cells displayed nuclear enlargement, minimal to slight nuclear contour irregularities, and enlargement of the nucleolus with minimal changes in cell membrane outline. Additionally, at medium concentration, cells demonstrated a mild to moderate increase in nuclear density, a further increase in nucleolar size, shrinking of cytoplasmic membranes, changes in cell size, and nuclear:cytoplasmic (N : C) ratio. At the highest concentration, nuclear hyperchromasia increased significantly with marked nuclear contour irregularities and inconspicuous nucleoli. A second population of large atypical cells with irregular and folded nuclei emerged in the exposed cultures constituting only 5–10% of the total population. These large cell population were excluded from the morphometric evaluation which showed that the N : C ratios for seven of the carcinogen exposures (nickel sulphate, chromium chloride, benzo(b)fluoranthene, indeno(1,2,3,-cd)pyrene, ethyl carbamate, N-nitrosodiethylamine, and NNK) were statistically significantly higher than controls at different carcinogen concentrations (Table 2). PAP-stained, vehicle-treated cells did not show any of the morphological changes described above (not shown). NSE staining was consistently absent in all twelve toxin-exposed cell groups at all concentrations with the exception of cadmium and chromium chloride, which showed weak activity at low (L) concentration. Intense peroxidase staining was observed in medium only control cells (N). Cells incubated with solvents (S) demonstrated NSE activity comparable to medium only controls (Figure 1).

*3.2. Phase Contrast Microscopy and Electron Microscopy.* All toxin-treated cell groups demonstrated shrinkage, small cell size, and nuclear granularity with membrane irregularity using phase contrast microscopy. A notable finding was cytoplasmic blebbing and outpouching with loss of cell membrane smoothness induced by N-nitrosodiethylamine even at low concentration (Figure 2). Cells treated with nickel sulphate, benzo(b)fluoranthene, and NNK revealed striking and consistent changes in the nucleoli by electron microscopy. Nucleolar size increased markedly with changes in shape including elongation, as well as multiple enlarged

Table 2: N : C ratios at 24-hour carcinogen exposure compared to vehicle controls.

| Carcinogen | N : C ratio at high dose | N : C ratio at med dose | N : C ratio at low dose |
|---|---|---|---|
| Nickel sulphate | 0.338 (0.033) | 0.318 (0.032) | 0.196 (0.034) |
| | $P = .002$ | $P = .017$ | $P = .042$ |
| Chromium chloride | 0.300 (0.031) | 0.365 (0.033) | 0.370 (0.033) |
| | NS | $P < .05$ | $P < .05$ |
| Benzo(b)fluoranthene | 0.360 (0.034) | 0.242 (0.033) | 0.253 (0.033) |
| | $P < .05$ | NS | NS |
| Indeno(1,2,3,-cd)pyrene | 0.184 (0.046) | 0.248 (0.033) | 0.209 (0.034) |
| | $P = .03$ | NS | NS |
| Ethyl carbamate | 0.254 (0.029) | 0.273 (0.028) | 0.254 (0.028) |
| | NS | NS | NS |
| N-Nitrosodiethylamine | 0.285 (0.028) | 0.276 (0.028) | 0.214 (0.029) |
| | NS | NS | NS |
| NNK | 0.283 (0.032) | 0.319 (0.035) | 0.260 (0.032) |
| | NS | $P = .009$ | NS |

NS: not statistically significant ($P \geq .05$).

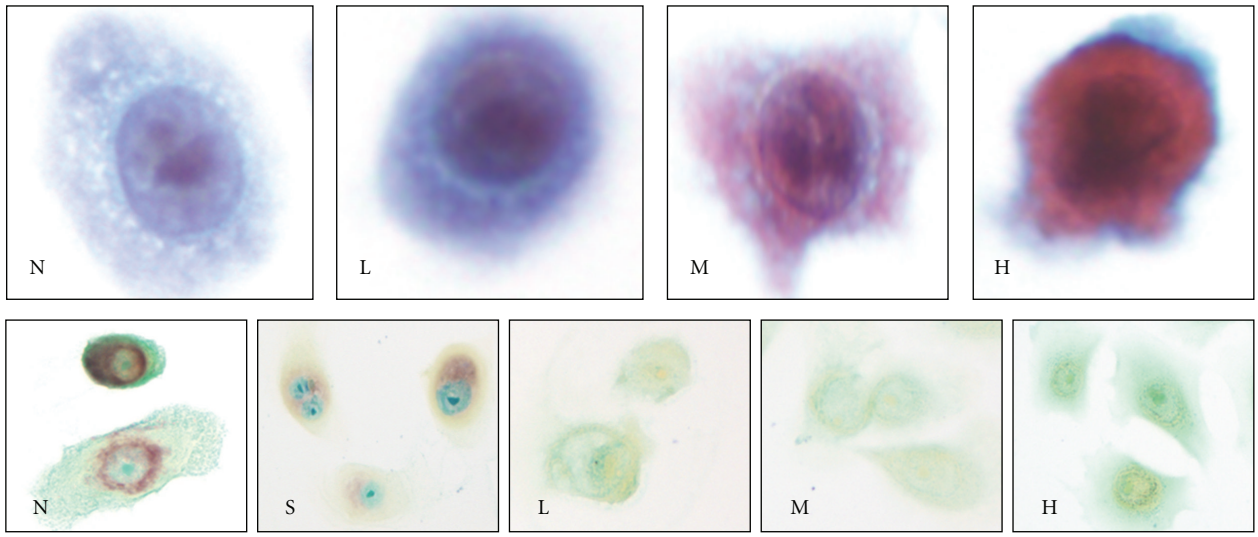


Figure 1: (60x magnification) Top Panel: effects of benzo(b)fluoranthene on morphology assessed by Papanicolaou stain. Note changes in cell size, nuclear size, nucleolar size, and nuclear and cytoplasmic density. Bottom Panel: histochemical staining for nonspecific esterase (NSE) of control and benzo(b)fluoranthene-treated cells. The negative (N) and solvent (S) controls (acetone exposure) display positive perinuclear, punctuate, or Golgi pattern of dense NSE staining while benzo(b)fluoranthene-exposed cells showed completely negative staining at all exposure concentrations. N: negative control, S: solvent control, L: low carcinogen concentration, M: medium carcinogen concentration, and H: high carcinogen concentration.

and/or irregularly shaped nucleoli. In some cells, the nucleolus appeared to span the inner diameter of the nucleus. Electron dense nonmembrane bound granular material was occasionally noted in the cytoplasm of cells not treated with toxins (Figure 3).

*3.3. Immunohistochemistry.* Negative control cells (media only or solvent) showed absent staining for all antibodies except membrane staining for *EGFR* (Figure 4). By omitting the primary antibody, "negative immunostaining controls" were also evaluated, none of which showed any staining (not shown). *BCL-2* staining at 24-hr exposure was negative in all cells except large atypical cells with multiple and irregular nuclei showing *BCL-2* localized to their nuclei with weak to moderate cytoplasmic staining. Cells with pyknotic and shrunken nuclei and those undergoing mitosis demonstrated strong nuclear positivity. These changes were consistent among all four toxins tested at both 24 and 48 hours; however, a few large atypical cells were positive at 48 hrs for *BCL-2*. *MKI67* was not evaluated at 48 hours. At 24-hour exposure, *MKI67* showed high reactivity in a speckled and granular pattern outlining the chromatin. In stark contrast to *BCL-2*, *MKI67* did not stain large atypical cells with irregular and multiple nuclei. Twenty large atypical

Table 3: Percent staining of *MKI67* in cultures exposed for 24 hours. All values expressed as percentages.

| *MKI67* | Percent (%) | | | |
|---|---|---|---|---|
| | Positive small cells | Negative small cells | Positive large cells | Negative large cells |
| Nickel sulphate | 93.6 | 6.3 | 0 | 20 |
| Benzo(b)fluoranthene | 92.6 | 7.3 | 0.3 | 19.6 |
| N-nitrosodiethylamine | 78 | 22 | 1 | 19 |
| NNK | 84.3 | 15.6 | 1 | 19 |

Table 4: Percent staining for *TP53*-positive cells.

| *TP53* | Percent (%) | | | |
|---|---|---|---|---|
| | Positive small cells | Negative small cells | Positive large cells | Negative large cells |
| Nickel sulphate 24 hr | 98.3 | 1.6 | 19 | 1 |
| Nickel sulphate 48 hr | 95.6 | 4.3 | 20 | 0 |
| Benzo(b)fluoranthene 24 hr | 68 | 32 | 19.3 | 0.6 |
| Benzo(b)fluoranthene 48 hr | 82.6 | 17.3 | 17.3 | 2.6 |
| N-nitrosodiethylamine 24 hr | 82.3 | 27.6 | 17.3 | 2.6 |
| N-nitrosodiethylamine 48 hr | 64.6 | 35.3 | 17.6 | 2.3 |
| NNK 24 hr | 92 | 8 | 17.6 | 2.3 |
| NNK 48 hr | 98.6 | 1.3 | 19.6 | 0.3 |

All values expressed as percentages.

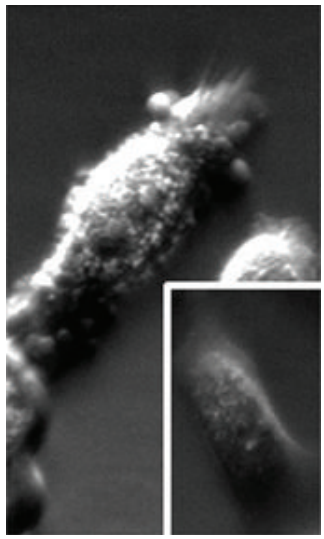

Figure 2: Although seen with many carcinogen exposures as occasional finding at higher concentrations, striking blebbing of the cytoplasmic membrane was noted with 24-hour N-nitrosodiethylamine exposure at extremely low concentration (1 ng/mL) detected by phase contrast microscopy at 60x. *Inset:* negative control as comparison with smooth and regular cell surface.

cell nuclei and 100 small-cell nuclei were counted three times and the average number of nuclei which were positively stained for *MKI67* was represented as a percentage of the total (Table 3). A similar approach was used to assess nuclear *TP53* staining in both small and large atypical cells (Table 4). Small cells stained positive while large cells were predominantly negative. In cells exposed to benzo(b)fluoranthene the number of *TP53* positive cells increased from 68% to 82.6% at the 24- versus 48-hour exposure interval. With N-nitrosodiethylamine, immunostaining for *TP53* was positive in a greater proportion of cells (82.3%) at 24 hours (82.3% versus 64.6%). Staining pattern of *TP53* remained the same at 24- and 48-hour exposures in the large atypical cells. At 24 hour exposure, *EGFR* demonstrated strong cytoplasmic and cell membrane staining and very weak nuclear staining in all cells. At 48-hour exposure, only nuclei of small cells stained strongly positive with no cytoplasmic or cell membrane staining. The large atypical cells with multiple irregular nuclei were mostly negative with occasional cells demonstrating weak and variable *EGFR* staining in the cytoplasm and/or nucleus. For all four toxins tested at 24- and 48-hour exposures, *BIRC5* activity was distributed primarily in the cytoplasm of cells, although some nuclear staining was also observed in cells exposed to nickel sulphate. Relatively stronger nuclear staining for *BIRC5* was noted in large atypical cells with irregular nuclei. At the 48-hour exposure, only cytoplasmic staining was observed in small and large cells. Some membranous staining in cells exposed to benzo(b)fluoranthene was noted. *BCL2L1* staining was present in both the cytoplasm and nucleus of the cells at 24- and 48-hour exposure. *BCL2L1* was more strongly positive in benzo(b)fluoranthene compared to nickel sulphate exposed cells at 24 hours (Figure 4). In general, there was no difference in cell viability between toxin treated cells and control cells (data not shown).

## 4. Discussion

In this study we have developed a reproducible technique for exposing human bronchial epithelial cells in culture to

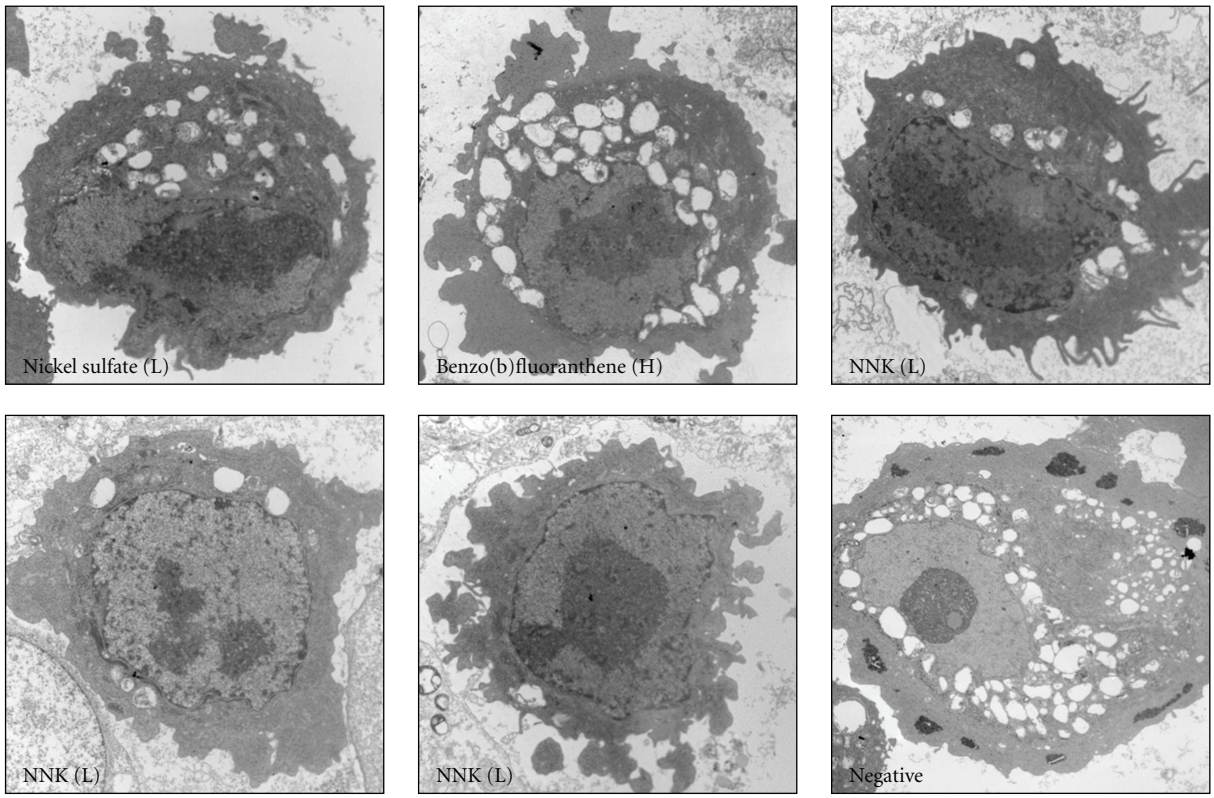


Figure 3: Electron microscopy shows striking and consistent nuclear and nucleolar changes with markedly increased size and changes in shape including elongation and multiple enlarged and/or irregularly shaped nucleoli and nucleolus appearing to span the inner diameter of the nucleus. The electron dense cytoplasmic granular material is present only in the negative control. NNK is represented in multiple images to show the pleomorphism in the toxin-induced nuclear changes. Direct magnification: 6500x, Print magnification: 11200x at 7 inch.

soluble tobacco toxins and have observed the early effects of these toxins on cell morphology, NSE activity, and selected gene expression. Although the immunohistochemical profile of various proteins in invasive lung carcinomas has been extensively studied, there are relatively few studies of protein expression in precancerous lesions [29], and virtually no information on the changes in cultured bronchial epithelial cells after toxin exposure. Among tobacco toxins benzo(a)pyrene (BaP), a prototypic polyaromatic hydrocarbon (PAH), has perhaps been the most extensively studied. The unique effects of other toxins are less well recognized in the complex composite milieu of tobacco smoke exposure experiments. There is limited data on exposure levels of individual tobacco toxins [30–36]. Accordingly, we chose a range of exposure concentrations based on the reported concentration of toxins found in a smoked cigarette [25]. In our experiments, cells were exposed for 24 and 48 hours, allowing adequate time for protein synthesis. Dibenz(a,h)anthracene and benzo(k)fluoranthene, which were soluble only in toluene, were excluded from our analysis to avoid the confounding issue of toluene cytotoxicity.

After exposure to toxins, cells displayed a spectrum of morphologic changes including nuclear enlargement and contour irregularities, enlargement of nucleoli, increase in nuclear density, shrinking of cytoplasmic membrane, changes in cell size, and N : C ratio, progressively accentuating from low to higher concentrations. In the exposed cell cultures, there emerged a second population of abnormal large atypical cells with very irregular and folded nuclei constituting 5–10% of the cellular population. All these features resemble those which characterize dysplastic cells. Our results further indicate a consistent decrease in NSE in toxin-treated cells relative to the controls for all tobacco toxins.

*EGFR* is overexpressed in human cancer cells and is linked to metastasis and resistance to treatment. In our study, *EGFR* was strongly positive in the cell membrane and cytoplasm with only weak nuclear staining following a 24-hour toxin exposure. After 48 hours of exposure, all *EGFR* staining was concentrated in the nucleus. The nonexposed cells showed only indistinct membranous rim-like staining. Immunostaining for *EGFR* has been shown to increase with the severity of dysplasia in preneoplastic and early neoplastic bronchial epithelium. Conversely, decreased expression of *EGFR* follows regression of bronchial squamous metaplasia [13, 16, 19, 29]. The nuclear shift of *EGFR* after a 48-hour toxin exposure correlates with the observation that, in response to growth factor stimulation, a fraction of *EGFR* moves from the cell surface to the nucleus, possibly interacting with STAT3 and directly regulating gene expression [15].

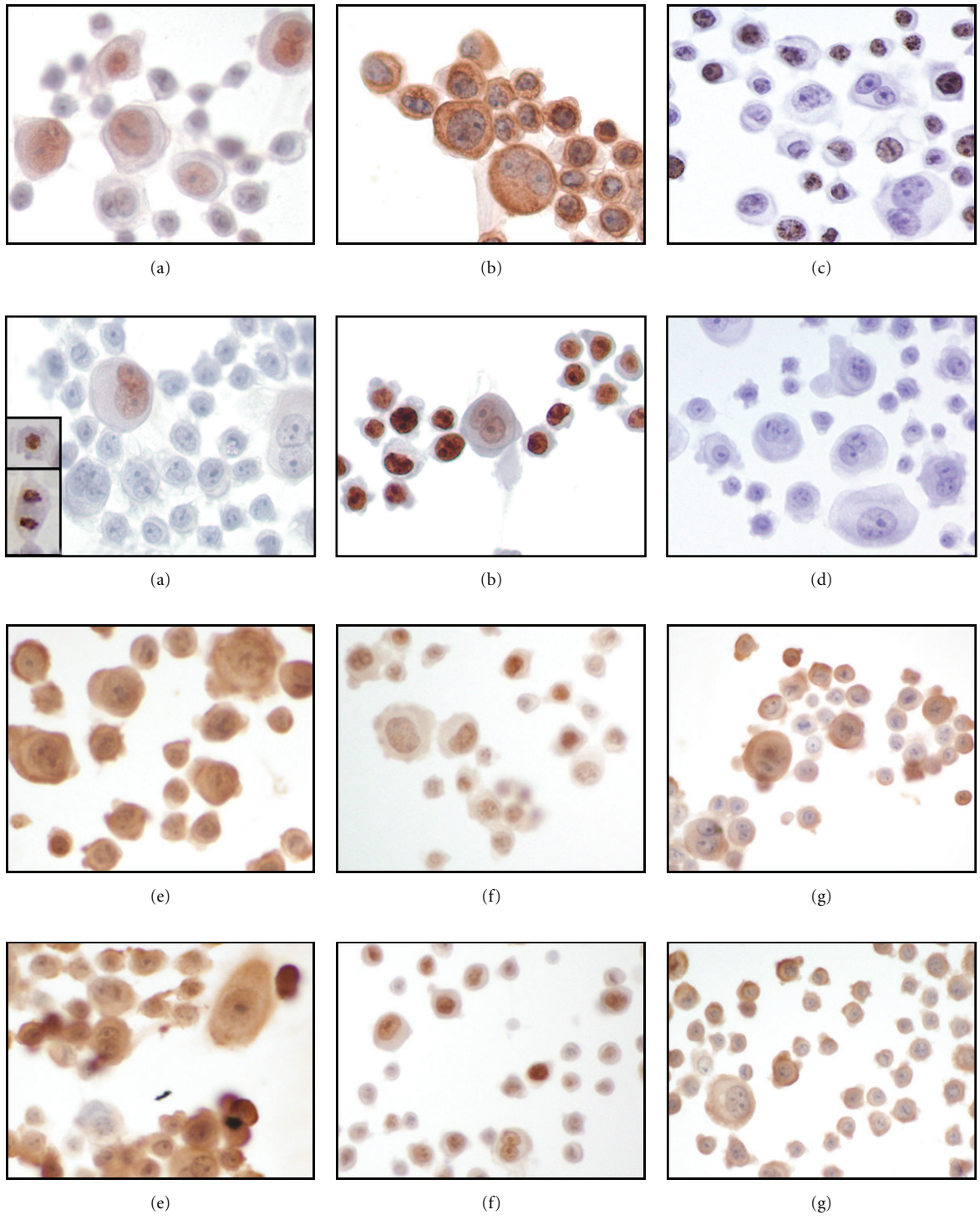


Figure 4: Immunostaining for *BCL-2*, *EGFR*, *Ki-67*, *BCL*-XL, p53, and BIRC5 after 24-hr (rows 1 and 3) and 48-hr (rows 2 and 4) exposure to benzo(b)fluoranthene at low concentration. Original magnification 60x. (a) *BCL*-2. Only nuclei of large atypical cells stain positively for *BCL*-2 at 24-hour exposure; staining is decreased slightly at 48-hour exposure. *Inset:* A pyknotic cell (top) and mitotic figure (bottom) stain strongly for Bcl-2. (b) *EGFR*. *EGFR* localized to cell membrane, cytoplasm, and weakly to nuclei of all cells at 24-hour exposure; staining shifts to the nuclei at 48-hour exposure, strongly in small cells and weakly in large atypical cells. (c) *MKI67*, assessed at 24-hr exposure only, strongly stained small cell nuclei with negative staining in large cells. (d) Negative control. Representative image of absence of immunoperoxidase staining. (e) *BCL2L1* Similar cytoplasmic and nuclear staining at both 24- and 48-hour exposure. F. *TP53*. Nuclear staining is noted in nearly all cells at 24-hour exposure, with increased intensity at 48-hour exposure. (g) *BIRC5* staining is localized mainly in the cytoplasm of cells following the 24-hour exposure with relatively stronger nuclear staining in large atypical cells. At 48-hour exposure, only cytoplasmic staining is observed with focal membranous staining.

*BCL-2* expression has been seen in less aggressive tumor behavior and is linked to increased cell survival [7]. Our results indicate absent immunostaining for *BCL-2* in the majority of cells exposed to toxins for 24 or 48 hours. However, the subpopulation of large atypical cells with irregular and large nuclei demonstrates positive nuclear and weak cytoplasmic *BCL-2* staining at 24 hours which persisted with decreased intensity at 48-hrs. In the unexposed cells, *BCL-2* staining was undetectable. Reported studies in tissue sections show basal *BCL-2* staining in normal epithelium, basal and suprabasal staining in metaplastic epithelium, and increasingly aberrant *BCL-2* expression with increasing grades of dysplasia [7–9]. Substantial pools of *BCL-2* have been identified within interphase nuclei controlling cellular proliferation that may induce rather than protect cells from apoptosis [8, 37]. Our results also suggest that *BCL-2* is expressed early after toxin exposure.

The fact that *MKI67* is present during all active phases of the cell cycle and undetectable in resting cells makes it an excellent marker for determining the growth fraction of a given cell population [22]. We found high *MKI67* immunostaining both speckled and granular patterns, outlining the nuclear chromatin material of toxin-exposed cells (Table 3). In contrast to *BCL-2*, immunostaining for *MKI67* was not seen in the population of large atypical cells with multiple or irregular nuclei. *MKI67* was completely absent in all cells in the unexposed cultures. Various studies have reported high *MKI67* activity in bronchial dysplastic lesions including 62.5–100% by Wang et al. [36], 1 to 60% by Tan et al. [20, 36], and 49% expression in small cell lung cancer specimens by Paik et al. [36, 38]. Our finding of high *MKI67* immunostaining in toxin-exposed cells is therefore similar to observations reported in tissue sections from dysplastic lesions.

Studies have reported negative *BIRC5* staining in normal bronchial epithelium, minimally atypical hyperplastic, and nonneoplastic lesions adjacent to tumors. Both nuclear and/or cytoplasmic *BIRC5* expression has been identified in metaplastic, dysplastic, and hyperplastic lesions with moderate dysplasia [18, 36]. The level of *BIRC5* correlates with the degree of dysplasia and is highest in carcinomas [19, 36]. *BIRC5* was found to be localized to the nucleus in 70% of early NSCLC's and both in the cytoplasm and nucleus in 54% of cases. Moreover, it was also identified in atypical mitotic figures and in giant multilobed neoplastic nuclei [39]. We observed *BIRC5* mainly in the cytoplasm of cells following the 24-hour exposure, with some nuclear staining in cells exposed to nickel sulphate and relatively stronger nuclear *BIRC5* in large atypical cells with irregular nuclei. After 48-hour exposure, only cytoplasmic staining was observed, with focal membranous staining in cells exposed to benzo(b)fluoranthene. This pattern in cultured bronchial cells of persistent cytoplasmic *BIRC5* immuno-reactivity and minimal nuclear reactivity after 48 hours of exposure to toxins somewhat differs from other tissue-based studies in which a predominance of nuclear immuno-reactivity has been reported. This discrepancy may relate to the short time interval of toxin exposure in our cell culture model or to the combination effect of multitoxin exposure in previous studies.

In our study, strong *TP53* nuclear staining was noted in the cells exposed to some toxins for 24 hours which further increased after 48 hours (Table 4). The variation of response kinetics to different toxins could be a reflection of a different mechanism of stimulating *TP53*. Altering signaling pathway through protein phosphorylation or direct modification of intermediate signaling compounds is usually very fast. On the other hand, alteration of mRNA expression or stability takes a longer time, albeit shorter than an epigenetic modification. In nature, the half-life of these compounds is long (2–>300 days). However, the half-life of these compounds was never examined in tissue culture. In the control cultures, only rare small cells showed positive *TP53* staining. Several studies demonstrate suppression of *BIRC5* by *TP53* [17]. The *TP53* immunostaining is reported to be infrequent in normal or metaplastic mucosa but may be seen in as many as 30% of cases of mild bronchial epithelial dysplasia [36]. Progressively increased suprabasal expression of *TP53* can be seen with increasing grades of dysplasia. The likelihood of invasive cancer has been positively correlated with the degree of *TP53* expression in bronchial epithelium from the same lung lobe [40], suggesting that *TP53* may have predictive value in assessing the biological behavior of preneoplastic endobronchial lesions. In another study, 41% of patients with dysplastic lesions showing >10% *TP53*-positive nuclei later developed lung cancer whereas only 23% of those with *TP53*-negative lesions progressed to cancer (positive and negative predictive value of 78% and 77%, resp.) [41]. Our results show that exposure to tobacco toxins results in appearance of significant *TP53* nuclear immuno-reactivity compared with unexposed (control) cells, and that the intensity of staining increases with the duration of exposure to tobacco toxins.

There is little information in the literature on the expression of *BCL2L1* in preneoplastic/dysplastic pulmonary lesions. Cytoplasmic expression of *BCL2L1* and elevated *BCL2L1* gene transcripts have been reported in 81.7% and 60% of lung cancers, respectively. Patients with tumors expressing *BCL2L1* showed shorter median survival compared to patients without *BCL2L1*-expressing tumors [11]. Either cytoplasmic or nuclear expression of *BCL2L1* was found in 81.5% and 30.4% of lung cancers, respectively. Nuclear *BCL2L1* expression correlated among all histologic types with TNM stage IV and the high expression of cytoplasmic *BCL2L1* (81.9%) in resected non-small cell lung cancers without any apparent influence on clinical outcome [10]. In our study, both cytoplasmic and nuclear *BCL2L1* staining was present at 24- and 48-hour exposure intervals. *BCL2L1* was more strongly positive in benzo(b)fluoranthene, exposed cells compared to nickel-sulphate-treated cells at 24 hours, suggesting that *BCL2L1* expression may be toxin dependent.

In summary, our studies describe the effects of known pulmonary tobacco toxins on an established cell line of human bronchial epithelial cells. Although the concentration of these toxins in cigarette smoke has been determined, we could only estimate the appropriate concentration for application to cells in tissue culture. Under the conditions of our experiments, we found that a brief exposure of cells to tobacco toxins produces consistent and reproducible morphologic changes of cell and nuclear size and shape.

Using immunohistochemistry we found that cells treated with toxins showed emergence of activities of *EGFR*, *BCL-2*, *MKI67*, *BCL2L1*, *BIRC5*, and *TP53* not found in untreated control cells. These changes are similar to those reported in tissue specimens of preneoplastic lesions and fully developed lung cancer. The findings of this study suggest that changes in expression of these proteins occur at a rapid rate after exposure of the cells to toxins, raising the possibility that some changes associated with overt malignancy might occur rapidly *in vivo* following toxin exposure. Prolonged or intermittent toxin exposure effect on cells is not known. Additional studies using human bronchial epithelial cell lines with other toxins or chronic intermittent exposure might increase our understanding of pathways involved in the development of lung cancer.

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*Research Article*

# Hypermethylation of CCND2 May Reflect a Smoking-Induced Precancerous Change in the Lung

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It remains unknown whether tobacco smoke induces DNA hypermethylation as an early event in carcinogenesis or as a late event, specific to overt cancer tissue. Using MethyLight assays, we analyzed 316 lung tissue samples from 151 cancer-free subjects (121 ever-smokers and 30 never-smokers) for hypermethylation of 19 genes previously observed to be hypermethylated in nonsmall cell lung cancers. Only APC (39%), CCND2 (21%), CDH1 (7%), and RARB (4%) were hypermethylated in >2% of these cancer-free subjects. CCND2 was hypermethylated more frequently in ever-smokers (26%) than in never-smokers (3%). CCND2 hypermethylation was also associated with increased age and upper lobe sample location. APC was frequently hypermethylated in both ever-smokers (41%) and never-smokers (30%). BVES, CDH13, CDKN2A (p16), CDKN2B, DAPK1, IGFBP3, IGSF4, KCNH5, KCNH8, MGMT, OPCML, PCSK6, RASSF1, RUNX, and TMS1 were rarely hypermethylated (<2%) in all subjects. Hypermethylation of CCND2 may reflect a smoking-induced precancerous change in the lung.

## 1. Introduction

Lung cancer causes more deaths in the United States each year than breast, colon, pancreatic, and prostate cancer combined, approximately 157,300 deaths estimated in 2010 [1]. Cigarette smoking is the most significant risk factor for developing lung cancer and contributes to 80–90% of these deaths [2, 3].

Over the past four to five decades, significant progress has been made to elucidate the carcinogenic mechanisms of tobacco smoking. Using animal models, it has been shown that among over 60 established carcinogens in cigarette smoke, 20 can cause lung tumors [4]. It has been proposed that these carcinogens, when metabolized, form DNA-adducts which may directly cause genetic alterations if not repaired. When these genetic alterations affect tumor suppressor genes or tumor oncogenes, they can promote cell proliferation and malignant transformation [5]. Studies in lung cancer patients clearly suggest that cigarette smoking can lead to acquisition of genetic mutations in p53 and ras oncogene [6, 7]. In addition, cigarette smoke is proposed to cause immunosuppression, which provides an environment for tumor progression [8, 9].

Recently, DNA hypermethylation has been recognized as an alternative, epigenetic mechanism for gene silencing in lung cancer, in addition to genetic mutation. Several environmental exposures are thought to cause aberrant DNA methylation, including dietary factors, chemotherapeutic

agents, and heavy metals [10]. Tobacco smoke exposure has been associated with increased expression of DNA methyltransferases [11–14]. Consistent with this observation, lung cancers arising in heavy smokers show increased hypermethylation of various genes, especially CDKN2A (p16) and RASSF1, compared with lighter smokers or nonsmokers [15–27].

However, these results do not reveal whether DNA hypermethylation occurs early or late in carcinogenesis. Early changes in carcinogenesis (especially those related to smoking) are hypothesized to occur somewhat diffusely in the lung and may therefore be detectable in noncancerous lung tissue, as well as in any cancers which arise [28–30]. For example, frequent hypermethylation of CDKN2A, RASSF1, CDH13, and other genes has been observed in sputum samples from cancer-free smokers, suggesting that they may be hypermethylated early [31–34]. In contrast, late changes in carcinogenesis are thought to arise mainly in overtly malignant tissues.

We recently analyzed matched cancerous and noncancerous lung tissues from patients with nonsmall cell lung cancer (NSCLC). We observed that in the 27 genes tested, most DNA methylation changes were tumor-specific and therefore might be considered late changes in carcinogenesis [35]. However, in these NSCLC patients, a small number of genes, including CCND2, APC, CDH1, and RARB (Table 1), were also hypermethylated in a portion of noncancerous lung tissues, suggesting that one or more of these genes might become hypermethylated as an early precancerous change. We hypothesized that early changes in DNA methylation, if present, might be associated with exposure to cigarette smoke. Furthermore, because smoking-related lung tumors and emphysema are known to disproportionately affect the upper lobes of the lungs [36, 37], we hypothesized that methylation changes related to smoking would similarly be more frequent in the upper lobes, compared with the lower lobes.

## 2. Materials and Methods

*2.1. Subject Enrollment.* All procedures were conducted in accordance with institutional review board and human subjects committee approval. Subjects were retrospectively enrolled who had undergone lung surgeries (lung volume reduction, lung transplant, wedge biopsy, or lobectomy) for nonmalignant diseases including emphysema, chronic bronchitis, bronchiectasis, granulomatous disease, various infectious diseases, and cystic or pulmonary fibrosis, at the University of Washington Medical Center (UWMC) between July 1st 1995 and July 1st 2005. All specimens were reviewed by an expert pathologist (CDJ) to confirm that they represented noncancerous lung tissue. All clinical data were gathered from subjects' UWMC medical records, including smoking history and primary pulmonary diagnosis. Subjects were excluded for the following reasons: previous diagnosis of lung cancer, insufficient lung tissue for methylation analysis, or unknown pack years of smoking. In total, 372 nonmalignant lung tissue samples from 159 subjects were identified for DNA methylation analysis.

*2.2. DNA Isolation from Paraffin Blocks.* From each block, six 20-$\mu$m sections were cut and deparaffined by xylene extraction. Proteinase K was used to digest the resulting tissue pellets overnight, at 48°C. Genomic DNA was then isolated by phenol/chloroform extraction and ethanol precipitation. Finally, DNA was purified using a QIAamp DNA minicolumn (Qiagen) according to the manufacturer's instructions.

*2.3. Sodium Bisulfite Conversion.* As previously described in detail [42], *in vitro* fully methylated DNA (methylated DNA control) and human sperm DNA (unmethylated DNA control) were converted with clinical samples. Briefly, ~1 $\mu$g DNA was modified by 5 mol/L sodium bisulfite, desulfonated with NaOH, and then purified and resuspended in 80 $\mu$L elution buffer (EB; 10 mmol/L Tris-HCl, pH 8.0).

*2.4. DNA Methylation (MethyLight) Assay.* All primers and probes for MethyLight assays were designed specifically for bisulfite-converted fully methylated DNA. Their sequences have been reported previously [35]. Amplification of bisulfite converted beta-actin (ACTB) DNA was used to normalize for the quantity of input DNA. Samples negative for ACTB were excluded from methylation analysis. Of 372 identified samples, 56 (15%) were excluded because they were negative for ACTB. The percentage of samples excluded after bisulfite conversion was similar in smokers (15%) and nonsmokers (16%). A plasmid containing bisulfite converted ACTB gene of known concentration was diluted and used as a standard curve for quantification. The assay for a given set of samples was only considered valid if the converted unmethylated human sperm DNA was not amplified, whereas the converted fully methylated DNA was amplified. For each locus, the percentage methylated reference (PMR) was calculated by dividing the gene/reference ratio of a sample by the gene/reference ratio of fully methylated DNA control [43]. Genes were considered to be positive for any hypermethylation at PMR >0%.

*2.5. Statistical Methods.* For comparisons between groups, to provide independent observations, we randomly selected one tissue block per subject to represent each subject's hypermethylation profile. To evaluate potential differences in gene methylation by site of the lung, paired upper, and lower lobe tissue samples from within subjects were compared using McNemar's Test. To assess the univariate and multivariate relationships between gene methylation and independent variables (smoking, age, gender, lobe of lung, pack years, and years since quitting), we included all available tissue samples from each subject and employed generalized estimating equations (GEE). This method enables the analysis of data with repeated measurements (multiple tissue samples per subject from different lobes) and accounts for within-subject correlations. In selecting a model, a logit link was used and we assumed an exchangeable working correlation structure to account for intrasubject correlation. Parameter estimates were exponentiated to provide odds ratios (OR) and 95% confidence intervals (CI). A 2-sided 0.05 test level determined statistical significance for all

Table 1: Genes hypermethylated in >2% of noncancerous lung tissues.

| HUGO acronym | Gene name | Function |
|---|---|---|
| APC | Adenomatous polyposis coli | Cell cycle: inhibits WNT signaling pathway, involved in spindle assembly and chromosome segregation, cell adhesion, and cell migration [38]. |
| CCND2 | Cyclin D2 | Cell cycle: regulates entry into S-phase with CDK4 and CDK6 [39] |
| CDH1 | Cadherin 1; e-cadherin (epithelial) | Cell adhesion, epithelial-mesenchymal transition [40] |
| RARB | Retinoic acid receptor, beta | Regulation of cell proliferation and differentiation [41] |

analyses. All analyses were conducted using SAS version 9.1 (SAS Institute Inc., Cary, NC).

## 3. Results

*3.1. Study Population and Tissue Samples.* We retrospectively enrolled 151 subjects who contributed a total of 316 available pathology blocks (Table 2). At the time of their surgery, 121 subjects were current or former smokers (ever-smokers), while 30 reported no smoking history (never-smokers). Among the never-smokers, none had any history of cancer, either prior to or after the surgery that yielded the tissue used in this study. Of the ever-smokers, 10 had a history of prior cancer other than lung (1 breast, 2 cervical, 2 colon, 2 prostate, 1 testicular, and 2 uterine) and all were cancer free at surgery. Four of the ever-smokers developed a cancer subsequent to the surgery that yielded the cancer-free lung tissue (1 bladder, 1 colon, and 2 NSCLCs, one at 2 years after, one at 5 years after). The clinical data show that never-smokers and ever-smokers who had undergone lung surgery comprised two distinct populations. Ever-smokers were significantly older than never-smokers (61 years versus 44 years). Further, of ever-smokers who contributed specimens from lung surgery, 71% had a diagnosis of emphysema, compared to only 10% of never-smokers.

We analyzed a total of 316 available pathology blocks from these 151 subjects, including 177 upper lobe samples, 105 lower lobe samples, 30 middle lobe or lingula samples, and 4 whose lobe of origin was unclear. Multiple blocks were available for 98 (81%) of ever-smokers and 13 (43%) of never-smokers; from the 121 ever-smokers, 269 samples were tested, while from the 30 never-smokers, 47 samples were tested. Sample sites varied substantially in ever-smokers and never-smokers as 50% of ever-smokers, compared to 17% of never-smokers, contributed only samples from the upper lobes. This difference arose because many ever-smokers in our sample underwent lung volume reduction surgery for emphysema, which predominantly affects the upper lung zones when induced by smoking.

*3.2. Gene Hypermethylation and Smoking Status.* Considering one random tissue block per subject, only APC (39%), CCND2 (21%), CDH1 (7%), and RARB (4%) were hypermethylated in more than 2% of subjects (Figure 1). All 15 remaining genes (BVES, CDH13, CDKN2A (p16), CDKN2B, DAPK1, IGFBP3, IGSF4, KCNH5, KCNH8, MGMT, OPCML, PCSK6, RASSF1, RUNX, and TMS1) were hypermethylated in less than 2% of subjects. CCND2 was hypermethylated significantly more frequently in ever-smokers compared to never-smokers (26% versus 3%, $P < .001$). APC was hypermethylated somewhat more frequently in ever-smokers (41% versus 30%), but this did not achieve statistical significance ($P = .3$).

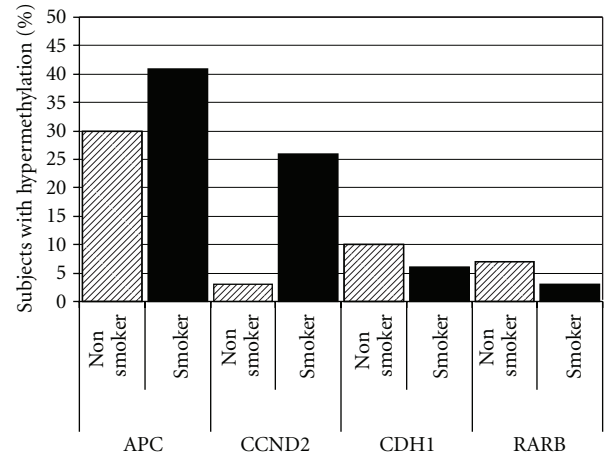


Figure 1: Hypermethylation of four genes in noncancerous lung tissues. Percent of subjects with hypermethylation of four genes (APC, CCND2, CDH1, and RARB), stratified by smoking status. Samples were considered to be positive for any hypermethylation at PMR >0%. To provide population statistics, one lung tissue sample per subject was randomly selected. The 15 other genes tested were hypermethylated in <2% of all subjects.

*3.3. Correlation of APC and CCND2 Gene Hypermethlation.* APC and CCND2 were often hypermethylated in the same samples; 179 (57%) samples were negative for both genes, 16 (5%) were positive for hypermethylation of CCND2, but not APC, 68 (22%) were positive for hypermethylation of APC but not CCND2, and 53 (17%) samples were positive for both genes. CCND2 hypermethylation was significantly correlated with APC hypermethylation in all subjects (OR = 7.3, 95% CI = 3.9–13.8) and in smokers only (OR = 7.4, 95% CI = 3.9–14.0). In nonsmokers, 31 (66%) samples were

Table 2: Clinical data of 151 cancer-free subjects with lung tissue available for MethyLight assay.

| | Never-smokers ($n = 30$) | Ever-smokers ($n = 121$) |
|---|---|---|
| *Age at surgery (mean years ± sd)* | 43.7 ± 11.6 | 61.0 ± 9.9 |
| 20–39 | 11 (37%) | 2 (2%) |
| 40–49 | 8 (27%) | 14 (12%) |
| 50–59 | 9 (30%) | 33 (27%) |
| 60–69 | 2 (7%) | 43 (36%) |
| 70–79 | 0 (0%) | 29 (24%) |
| *Female gender* | 18 (60%) | 58 (48%) |
| *Smoking pack years* | | |
| 1–39 | N/A | 50 (41%) |
| ≥40 | N/A | 71 (59%) |
| *Years since quitting*[a] | | |
| 0 (Current) | N/A | 15 (13%) |
| 1–4 | N/A | 31 (26%) |
| 5–9 | N/A | 27 (23%) |
| 10–19 | N/A | 30 (25%) |
| ≥20 | N/A | 17 (14%) |
| *Surgery* | | |
| Lung volume reduction | 0 (0%) | 57 (47%) |
| Lung transplant | 9 (30%) | 31 (26%) |
| Wedge Biopsy | 18 (60%) | 24 (20%) |
| Lobectomy | 2 (7%) | 5 (4%) |
| Bullectomy | 0 (0%) | 4 (3%) |
| Segmentectomy | 1 (3%) | 0 (0%) |
| *Number of samples evaluated* | | |
| One sample | 17 (57%) | 23 (19%) |
| Multiple samples | 13 (43%) | 98 (81%) |
| *Sample locations*[b] | | |
| Upper lobe only | 5 (17%) | 60 (50%) |
| Middle lobe or lingula only | 6 (21%) | 1 (1%) |
| Lower lobe only | 10 (34%) | 21 (18%) |
| Multiple lobes | 8 (28%) | 37 (31%) |
| *Etiology* | | |
| Emphysema[c] | 3 (10%) | 86 (71%) |
| Inflammatory conditions[d] | 13 (43%) | 21 (17%) |
| Infectious diseases | 4 (13%) | 7 (6%) |
| Cystic fibrosis | 5 (17%) | 0 (0%) |
| Pulmonary hypertension | 1 (3%) | 2 (2%) |
| Sarcoidosis | 1 (3%) | 1 (1%) |
| Lymphoid hyperplasia | 1 (3%) | 1 (1%) |
| Infarct | 1 (3%) | 1 (1%) |
| Hemangioma | 0 (0%) | 1 (1%) |
| Trapped lung | 1 (3%) | 0 (0%) |
| No histologic abnormalities | 0 (0%) | 1 (1%) |

[a] Quit years not available for 1 subject.

[b] Sample location unknown for 4 samples from 3 subjects.

[c] See results section for details.

[d] Inflammatory conditions included chronic bronchitis, bronchiectasis, pulmonary fibrosis, and granulomatous disease.

Table 3: All subjects—odds ratios for promoter hypermethylation (95% CI).

| | Ever-smokers versus never-smokers | Age per 10 years | Female versus male | Upper versus lower lobe |
|---|---|---|---|---|
| Univariate[a] | | | | |
| APC | 1.3 (0.6–2.9) | 1.1 (0.9–1.4) | 0.6 (0.5–0.8) | 1.6 (0.9–2.7) |
| CCND2 | 6.9 (1.6–29.8) | 1.9 (1.4–2.7) | 0.8 (0.6–1.1) | 2.3 (1.2–4.4) |
| Multivariate[a] | | | | |
| APC | 1.0 (0.4–2.6) | 1.0 (0.8–1.3) | 0.6 (0.4–0.8) | 1.6 (1.0–2.8) |
| CCND2 | 2.8 (0.6–12.1) | 1.7 (1.2–2.4) | 0.8 (0.6–1.2) | 2.0 (1.0–3.8) |

[a] Associations between clinical parameters and gene hypermethylation, assessed at PMR > 0%, in all 316 lung specimens from 151 subjects.

negative for both genes, 14 (30%) were positive for APC only, and 2 (4%) samples were positive for both APC and CCND2.

*3.4. Gene Hypermethylation and Clinical and Demographic Factors.* In univariate GEE analyses of all specimens (Table 3), CCND2 hypermethylation was significantly associated with a positive smoking history, increasing age, and sample origin from the upper versus lower lobe of the lung. APC hypermethylation was significantly less frequent among females and moderately more frequent in upper lobes compared to lower lobes but was not significantly associated with a positive smoking history. In a multivariate model simultaneously assessing smoking history, age, gender, and location of the sample (upper versus lower lobe) in all subjects, hypermethylation of CCND2 remained significantly associated with increased age (OR = 1.7, 95% CI = 1.2–2.4 for each 10 years of age) and upper lobe location (OR = 2.0, 95% CI = 1.0–3.8). CCND2 hypermethylation was somewhat associated with a positive smoking history (OR = 2.8, 95% CI = 0.6–12.1) but this did not achieve statistical significance.

*3.5. Gene Hypermethylation and Duration of Smoke Exposure.* Within the subset of 269 samples from 121 ever-smokers (Table 4), APC hypermethylation was not related to pack-years of cigarette smoking or years since quitting smoking. In univariate GEE analysis, CCND2 hypermethylation was significantly associated with greater pack years but was not related to years since quitting. However, in a multivariate GEE analysis simultaneously assessing pack years, years since quitting, age, gender, and location of the sample (upper versus lower lobe), CCND2 hypermethylation was no longer associated with pack years (OR = 1.0, 95% CI = 0.9–1.2 per 10 pack years).

*3.6. Gene Hypermethylation in Upper- and Lower-Lobe Samples.* Smoking-related lung tumors and emphysema are known to disproportionately affect the upper lobes of the lungs. Thus, if hypermethylation of a gene is associated with smoking, we might expect to find more hypermethylation in upper lobe samples compared to lower lobe samples, among ever-smokers.

Examining all 269 samples from ever-smokers (Table 4), in univariate GEE analysis, both APC (OR = 2.0, 95% CI = 1.1–3.5) and CCND2 (OR = 1.9, 95% CI = 1.0–3.5) hypermethylation were more common in upper compared to lower lobes. In multivariate analysis including pack-years, years since quitting, age, gender, and upper versus lower lobe, APC hypermethylation remained significantly associated with upper-lobe sample location (OR = 2.1, 95% CI = 1.1–4.0), while CCND2's positive association with upper lobes was reduced to slightly below the level of statistical significance (OR = 1.7, 95% CI = 0.9–3.4).

Among the 121 ever-smokers in our cohort, 30 had both upper and lower lobe samples available, and were included in within-subjects, pairwise comparisons. For APC, 12 of 30 pairs had discordant hypermethylation status (1 positive and 1 negative), of which 8 of 12 displayed APC hypermethylation in an upper lobe but not a lower lobe sample ($P = .25$). For CCND2, only 7 of 30 pairs had discordant hypermethylation status, of which only 3 of 7 were hypermethylated in the upper but not the lower lobe ($P = .7$). Thus, too few subjects had discordant hypermethylation in upper and lower lobe samples to yield statistically meaningful results in within-subjects comparisons.

## 4. Discussion

DNA hypermethylation is an important event in lung carcinogenesis. However, it is currently unknown whether changes in DNA methylation are early events, occurring in previously normal lung tissue or whether they are late changes that occur only in overt tumor cells [30]. To attempt to answer these questions, we tested DNA hypermethylation in lung tissues from subjects without cancer—both smokers and nonsmokers—using a panel of 19 genes which we had previously found to be hypermethylated in some nonsmall cell lung cancers [35, 44]. This unique study design allowed us, for the first time, to characterize the DNA hypermethylation profile of nonsmokers' lung tissues and to compare this profile to that of smoke-exposed lung.

Importantly, we observed that CCND2, which is known to be frequently hypermethylated in lung cancer tissue [35, 44–47], was hypermethylated more frequently in ever-smokers (26%) than in never-smokers (3%). Also, as predicted, in ever-smokers, CCND2 was hypermethylated more frequently in samples from the upper lobes, which are known to suffer far more negative effects from cigarette smoke, such as lung cancer and emphysema [36, 37]. These findings support the conclusion that CCND2 reflects an early, precancerous change in the lung, caused by cigarette smoke.

Table 4: Ever-smokers only—odds ratios for promoter hypermethylation (95% CI).

| | Pack years per 10 years | Quit years per 10 years | Age per 10 years | Female versus male | Upper versus lower lobe |
|---|---|---|---|---|---|
| Univariate[a] | | | | | |
| APC | 1.0 (0.9–1.1) | 0.9 (0.6–1.2) | 1.1 (0.8–1.5) | 0.6 (0.5–0.8) | 2.0 (1.1–3.5) |
| CCND2 | 1.1 (1.0–1.3) | 0.8 (0.6–1.1) | 1.8 (1.3–2.6) | 0.8 (0.6–1.1) | 1.9 (1.0–3.5) |
| Multivariate[a] | | | | | |
| APC | 0.9 (0.8–1.1) | 0.8 (0.6–1.2) | 1.2 (0.8–1.7) | 0.6 (0.4–0.8) | 2.1 (1.1–4.0) |
| CCND2 | 1.0 (0.9–1.2) | 0.8 (0.5–1.2) | 1.8 (1.2–2.9) | 0.9 (0.6–1.2) | 1.7 (0.9–3.4) |

[a] Associations between clinical parameters and gene hypermethylation in 269 lung specimens from 121 subjects with a current or past history of smoking.

CCND2 encodes cyclin D2, a protein involved in cell cycle progression that is thought to act as a regulator of cyclin dependent kinase 4 and cyclin dependent kinase 6 in the transition from G1 to S-phase [39]. CCND2 hypermethylation appears to be common in many cancers. In breast cancer, where it has been studied most extensively, CCND2 hypermethylation is detected frequently, though it appears to be rarely detected in normal breast tissue [48–54]. Interestingly, while CCND2 hypermethylation (and therefore low CCND2 protein expression) has been associated with poor prognosis in epithelial ovarian cell cancer [55] and recurrence of hepatocellular carcinoma [56], *increased* CCND2 expression has been associated with poor prognosis in diffuse large B-cell lymphoma [57].

In the lung, CCND2 hypermethylation has been found in 40–56% of NSCLCs [35, 44, 45, 47]. In noncancerous lung tissue, whereas Virmani et al. found CCND2 hypermethylation in 0 of 18 samples [45], our previous investigation found CCND2 hypermethylation in 24% of noncancerous lung tissues from patients with NSCLC [35]. This closely matches the rate observed in the present study, in cancer-free ever-smokers (26%). Possibly, our group observed a higher rate of CCND2 hypermethylation in both cancerous and cancer-free lung tissues because we used MethyLight assays instead of methylation-specific PCR (MSP), which was used by Virmani et al. Thus, we may have detected low levels of hypermethylated genes in cancer-free tissues which were not detected by MSP. Discrepancies may also be due to the somewhat different primers and probes used in analyses, which indicate different sequence regions investigated. In addition, Kubo et al. did not observe any CCND2 hypermethylation in 30 matched noncancerous lung tissues but it should be noted that in this study, 70% of subjects were nonsmokers who would not be expected to have significant rates of CCND2 hypermethylation [46].

Combined, these results reveal a progression in the rate of CCND2 hypermethylation in the lung, corresponding with the risk for developing lung cancer. While CCND2 hypermethylation was very infrequent (3%) in our current study's low-risk group of 30 never-smokers, it was more frequent in a high-risk group of ever-smokers (24–26% in our current and previous studies), and most frequent in overt NSCLC tissue (40–56%). This risk-stratified progression in lung tissues suggests that CCND2 hypermethylation may truly reflect an early precancerous change in the lung, en route to overt cancer, which may be due to the effects of smoking.

Still, our findings regarding CCND2 should be regarded as preliminary at this time, for several reasons. In multivariate analysis, the effect of smoking status on CCND2 hypermethylation was reduced to trend-level significance after taking into account the effects of sample location (upper versus lower lobe) and subject age. This likely occurred because in our sample, the majority of smokers underwent lung surgery for emphysema and represented a significantly older group, more likely to contribute samples from upper lobes (where emphysema is most prominent). In contrast, nonsmokers were younger and underwent lung resection for a variety of diseases. With such significant correlation of these factors, multivariate analysis may not have reliably separated each factor's relative contribution to gene hypermethylation. Thus, observed differences in the rate of CCND2 hypermethylation could be attributable to any of these factors or others that differed between ever and never-smokers. Emphysema, for example, made CCND2 hypermethylation more likely although significant rates of CCND2 hypermethylation were also found in smokers with other diagnoses. While CCND2 hypermethylation could be part of the unique pathophysiology of emphysema, it more likely arose because emphysema reflects severe smoking-induced lung damage. The effect of age on CCND2 hypermethylation has not been studied previously in noncancerous lung, although several genes have been reported to undergo increased rates of hypermethylation with age, in various body tissues, including CDH1 and DAPK1 in the lung [58]. In noncancerous breast epithelium [59] and in peripheral blood samples from cancer-free subjects [60], advanced age was not observed to correlate with CCND2 hypermethylation. Thus, the relationship between age and CCND2 hypermethylation remains unknown at this time. In weighing the relative contributions of age, sample location, and emphysema status on CCND2 hypermethylation, it is worth noting that smoking history was by far the strongest single predictor of CCND2 hypermethylation in univariate analysis (OR = 6.9, 95% CI = 1.6–29.8). One limitation of the present study was that despite our overall large number of 151 subjects, only 30 were never-smokers. This occurred because never-smokers far less frequently undergo lung resections which produce tissue. This may have been part of the reason why in multivariate analysis, we observed only trend-level significance

for smoking's effect on CCND2 hypermethylation. We were able to improve our statistical power somewhat by using generalized estimating equations (GEE) for our univariate and multivariate analyses, allowing us to enter multiple tissue blocks per subject when available (multiple observations), without biasing the results. However, future studies should seek to verify the low rate of CCND2 hypermethylation we observed in never-smokers. An additional limitation of our study design was that all subjects had an underlying noncancer pulmonary diagnosis that necessitated lung surgery. Thus, while observed gene hypermethylation was unrelated to cancer, it cannot definitely be said to represent healthy lung. Finally, due to our study design, we only provide indirect evidence of interaction between smoking and CCND2 hypermethylation. Future studies utilizing animal models may be useful to elucidate the potential causal relationship between smoking and CCND2 hypermethylation.

In our current and previous studies, CDKN2A (p16) was hypermethylated in 26% of cancer tissues [44] but was rarely hypermethylated in noncancerous lung tissues, regardless of smoking status [35]. However, CDKN2A hypermethylation has previously been characterized as an early event in lung carcinogenesis [28–30], and hypermethylation of CDKN2A has been commonly detected in sputum samples from heavy smokers without lung cancer [32, 61]. Overall, a very wide range of hypermethylation rates for CDKN2A has been reported in the literature, for noncancerous lung tissues. Along with other researchers who observed low rates of CDKN2A hypermethylation in noncancerous lung tissues, our results suggest that CDKN2A hypermethylation may actually represent a later change in carcinogenesis [62–66]. However, the surprisingly large discrepancies between studies may be related to differences in assay methodology (including PCR primers and specific CpG islands) or patient populations.

## 5. Conclusions

CCND2 hypermethylation likely represents an early, smoking-induced, precancerous change in the lung; it is very infrequent in the lung tissue of never-smokers, more frequent among smokers, and most frequent in overt NSCLC tissue. This conclusion should be verified in future investigations. In addition, this study supports the conclusions of our previous investigation, that although they are hypermethylated in many NSCLC tumor tissues, RASSF1, DAPK1, BVES, CDH13, MGMT, KCNH5, and to some extent CDH1 and RARB, are rarely hypermethylated in the cancer-free lung, even after significant tobacco exposure [35]. These genes may therefore yield clues to understanding the later stages of carcinogenesis. In addition, if hypermethylation of CCND2 or other genes represents an early precancerous change, it is possible that drugs aimed at reversing DNA methylation could be used to prevent smoking-related carcinogenesis.

## Conflict of Interests

None Declared.

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*Research Article*

# Epidemiology of Cigarette and Smokeless Tobacco Use among South Asian Immigrants in the Northeastern United States

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As the most preventable cause of death in the world today, understanding tobacco use among one of the fastest growing ethnic/racial groups is warranted. We explore cigarette and smokeless tobacco (SLT) use among South Asians in NJ and the Northeast using the Tobacco Use Supplement to the Current Population Survey. Overall, tobacco use rates among South Asians were similar or lower than the population. However, in NJ, South Asian males had the highest SLT rate (2.7%) and in the Northeast, White (AOR = 5.8, 95% CI = 3.7–9.4) and South Asian males (AOR = 4.0, 95% CI = 1.5–10.6) had significantly higher odds of current SLT use relative to non-White males. Tobacco use among South Asians was not homogeneous; Pakistanis are overrepresented among cigarette smokers while Indians are overrepresented among SLT users. Given the differential tobacco use among and within South Asian, disaggregating data to understand tobacco use behaviors is necessary to develop effective interventions for tobacco cessation.

## 1. Introduction

Tobacco is the single most preventable cause of death in the world today, including South Asian countries like India where there are disparities in chronic diseases like cancer and cardiovascular disease that have surpassed infectious disease as the leading causes of death. South Asians are the third largest Asian group in the United States, comprising 1.89 million people and are among the fastest growing racial groups in New Jersey and the Northeast [1]. In 2000, one out of three South Asians reside in the Northeast, and there were almost 170,000 South Asians living in New Jersey, representing the 3rd largest statewide South Asian population in the country with the large majority of South Asian immigrants coming from India, Pakistan, Bangladesh, and Sri Lanka [1].

Despite marked health disparities in South Asians internationally compared to the US population (e.g., cancer, heart disease, and diabetes) [2–4], little is known about the health status of South Asians residing in the US Paradoxically, the South Asian population in the US is generally viewed as a successful immigrant group, resulting in a characterization known as the "Model Minority Myth." This concept describes a minority ethnic, racial, or religious group whose members achieve a higher degree of success, affluence, and thus good health, than the population as a whole. However,

recent data strongly contradict the notion that South Asians are uniformly affluent and healthy and highlight the growing heterogeneity of this group [1, 2, 5, 6].

Indeed, India is the second largest consumer of tobacco in the world, and national data indicate that 47% of men and 14% of women either smoke or chew tobacco [7]. Likewise, nearly one out of three adults in Bangladesh use some form of tobacco [8], and one out of three Pakistani males use tobacco daily [9]. Studies conducted in the UK suggest that South Asians who immigrate may have lower rates of smoking overall than the general population [10, 11]. However, other studies have found high rates of smoking in certain subgroups of South Asians, particularly Bangladeshis [12, 13]. Studies of tobacco use in South Asian immigrants in the US are limited and not applicable to the general population for two main reasons. First, studies have been geographically limited to a community, city, or single state [14–17]. Second, despite the traditional role of smokeless tobacco in South Asian cultures, some studies have focused only on cigarette smoking [2, 16, 17].

The paucity of research on tobacco use behaviors in South Asians is due in part to the fact that despite a distinct cultural and geographical background, South Asians are almost always aggregated into a broad "Asian" category, thus potentially masking subgroup differences and preventing identification of potential health disparities between subgroups. However, given that the explosive growth in the South Asian population is fairly recent and largely attributed to immigration, it is methodologically possible to identify South Asians in the Tobacco Use Supplement to the Current Population Survey (CPS), as the survey collects country of origin. The current study is the first to use population level behavioral surveillance data to explore patterns of cigarette and smokeless tobacco use among South Asians residing in New Jersey and the Northeast US and to explore tobacco use behavior by country of origin.

## 2. Methods

*2.1. Data Source.* We analyzed New Jersey and Northeast specific data from the 2003 and the 2006/7 Tobacco Use Supplement to the Current Population Survey (TUS-CPS). The details of the TUS-CPS sampling design and data collection methods are provided elsewhere [18]. In brief, the TUS-CPS uses an area probability sampling design to select a stratified probability sample of clusters of households. Approximately 56,000 households are surveyed in a given month using computer-assisted telephone interviewing (CATI) and computer-assisted personal interviewing (CAPI) methods. State estimates may be generated from the national TUS by combining multiple months of data. Individual level self-response rates for the TUS-CPS questionnaire are approximately 65–72% for those households completing the basic CPS household survey (response rates range from 93 to 97%). To increase sample size, we merged data from 2003 and 2006/7 for our analyses. The overall sample size for New Jersey was 7,354, of which 176 were South Asian, and for the Northeast there were 71,152 total cases of which 583 were South Asian.

*2.2. Race/Ethnicity, Country of Origin, and Immigrant Status Measures.* We constructed a single, five-level variable for race/ethnicity which included White, Black, Hispanic, Asian, and South Asian. This was constructed from five survey questions: race, Hispanic origin, country of origin, mother's country of origin, and father's country of origin. South Asians were defined as those individuals who indicated that their country of origin or the country of origin for one of their parents were from India, Pakistan, or Bangladesh. While the TUS did not permit the identification of other South Asian countries of origin (e.g., Sri Lanka, Nepal), the three countries we could identify comprise 98.5% of South Asian immigrants in the US [1]. In addition, we created an additional variable for South Asians only that reflected their immigration status and country of origin which resulted in four mutually exclusive categories: first generation American of South Asian descent (i.e., born in the US, but at least one parent is from India, Pakistan, or Bangladesh), immigrant from India, immigrant from Pakistan, and immigrant from Bangladesh.

*2.3. Tobacco Measures.* We applied standard tobacco surveillance criteria for calculating adult tobacco use prevalence [19]. Our measures of cigarette smoking were derived from three questions resulting in two measures: ever smoker (i.e., smoked 100 cigarettes in their lifetime), and current smoker (i.e., smoked 100 cigarettes and now smokes everyday or some days). With respect to smokeless tobacco, the TUS-CPS does not include a lifetime threshold question (e.g., smoked 100 cigarettes) for smokeless tobacco use, but does inquire about snuff tobacco and chew tobacco separately. Our measures of smokeless tobacco use were derived from four questions resulting in two measures: ever smokeless user (i.e., has used snuff or chew), and current smokeless user (i.e., now uses snuff or chew everyday or some days).

*2.4. Analysis.* Sample replicate weights were applied to adjust for nonresponse and the varying probabilities of selection, including those resulting from oversampling, providing results representative of New Jersey and the Northeast's adult population. SUDAAN statistical software, which corrects for the complex sample design, was utilized to generate point estimates and adjusted odds ratios (AOR) with 95% confidence intervals [20].

## 3. Results

Overall, 74.8% of adults in the Northeast were White, 10.5% were Black, 9.7% were Hispanic, 3.6% were Asian/PI (not South Asian descent), and 1.3% were South Asian. As shown in Table 1, South Asians are demographically different than their White, Black, Hispanic, and Asian counterparts. First, South Asians were more likely to be male (60.0%) compared to all other racial/ethnic groups and to the overall sample (47.5%). With respect to age, South Asians had a lower proportion of adults over the age of 65 compared to Whites, Blacks, and Asians. South Asians also had the largest proportion of adults with at least a college

Table 1: Demographic characteristics among adults in the US northeast by race/ethnicity, 2003–2006/7 Tobacco Use Supplement to the Current Population Survey.

| (Unweighted $n$) | White $n = 60{,}505$ | | Black $n = 4{,}449$ | | Hispanic $n = 4{,}485$ | | Asian $n = 1{,}442$ | | South Asian $n = 585$ | | Overall $n = 71{,}466$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % | 95% CI | % | 95% CI | % | 95% CI | % | 95% CI | % | 95% CI | % | 95% CI |
| *Gender* | | | | | | | | | | | | |
| Male | 47.7 | (47.5–47.9) | 44.0 | (43.3–44.6) | 48.1 | (46.7–49.4) | 46.3 | (44.0–48.6) | 59.9 | (56.2–63.7) | 47.5 | (47.4–47.6) |
| Female | 52.3 | (52.1–52.5) | 56.0 | (55.4–56.7) | 51.9 | (50.6–53.3) | 53.7 | (51.4–56.0) | 40.1 | (36.3–43.8) | 52.5 | (52.4–52.6) |
| *Age group* | | | | | | | | | | | | |
| 18–24 | 9.6 | (9.2–9.9) | 14.8 | (13.6–16.1) | 15.6 | (14.3–16.9) | 10.8 | (8.9–12.7) | 9.6 | (6.2–13.0) | 10.7 | (10.4–11.0) |
| 25–44 | 35.6 | (35.2–36.0) | 41.5 | (40.2–42.8) | 50.0 | (48.4–51.6) | 48.5 | (45.7–51.2) | 59.9 | (54.1–65.6) | 38.4 | (38.1–38.7) |
| 45–64 | 34.7 | (34.2–35.1) | 30.0 | (29.0–31.0) | 26.2 | (24.9–27.6) | 29.4 | (26.9–31.8) | 24.3 | (20.2–28.4) | 33.0 | (32.7–33.4) |
| 65+ | 20.2 | (19.8–20.6) | 13.7 | (12.7–14.6) | 8.2 | (7.2–9.3) | 11.4 | (9.1–13.7) | 6.3 | (3.8–8.7) | 17.8 | (17.5–18.2) |
| *Education* | | | | | | | | | | | | |
| Less than HS | 9.4 | (9.0–9.7) | 19.4 | (17.9–20.9) | 36.5 | (34.5–38.5) | 12.0 | (9.7–14.3) | 5.1 | (2.3–7.8) | 13.1 | (12.7–13.5) |
| HS | 32.9 | (32.3–33.5) | 34.6 | (33.0–36.2) | 30.0 | (28.5–31.6) | 21.0 | (18.3–23.8) | 14.2 | (10.4–18.0) | 32.1 | (31.6–32.7) |
| Some college | 23.6 | (23.1–24.1) | 26.1 | (24.7–27.6) | 19.3 | (18.0–20.7) | 15.6 | (13.5–17.7) | 10.0 | (6.8–13.2) | 23.0 | (22.6–23.4) |
| College | 34.1 | (33.3–34.8) | 19.9 | (18.5–21.3) | 14.1 | (12.8–15.5) | 51.4 | (47.6–55.1) | 70.8 | (65.6–75.9) | 31.8 | (31.1–32.4) |
| *State* | | | | | | | | | | | | |
| Connecticut | 6.6 | (6.5–6.8) | 5.1 | (4.7–5.4) | 5.8 | (4.8–6.7) | 3.7 | (2.7–4.7) | 8.1 | (4.9–11.3) | 6.3 | (6.3–6.3) |
| Maine | 3.2 | (3.2–3.2) | 0.2 | (0.1–0.2) | 0.2 | (0.1–0.3) | 0.4 | (0.3–0.6) | 0.2 | (0.0–0.3) | 2.4 | (2.4–2.5) |
| Massachusetts | 13.4 | (13.1–13.6) | 5.9 | (5.4–6.3) | 7.9 | (6.4–9.4) | 11.6 | (8.6–14.5) | 7.7 | (4.7–10.7) | 11.9 | (11.8–12.0) |
| New Hampshire | 3.0 | (3.0–3.1) | 0.2 | (0.1–0.2) | 0.4 | (0.3–0.6) | 1.1 | (0.6–1.5) | 1.1 | (0.5–1.7) | 2.4 | (2.4–2.4) |
| New Jersey | 13.8 | (13.5–14.1) | 19.0 | (18.2–19.8) | 21.9 | (19.9–23.8) | 21.6 | (18.4–24.7) | 34.8 | (27.7–41.9) | 15.7 | (15.6–15.7) |
| New York | 30.2 | (29.8–30.6) | 48.8 | (47.7–49.9) | 54.4 | (52.0–56.8) | 51.9 | (47.5–56.2) | 37.7 | (31.2–44.3) | 35.4 | (35.3–35.5) |
| Pennsylvania | 26.1 | (25.9–26.4) | 20.0 | (19.3–20.8) | 7.3 | (5.9–8.7) | 8.3 | (5.9–10.7) | 9.6 | (6.3–13.0) | 22.8 | (22.7–22.9) |
| Rhode Island | 2.2 | (2.1–2.2) | 0.8 | (0.7–0.9) | 2.1 | (1.7–2.4) | 1.3 | (0.9–1.7) | 0.5 | (0.1–0.8) | 2.0 | (2.0–2.0) |
| Vermont | 1.5 | (1.5–1.5) | 0.1 | (0.1–0.1) | 0.1 | (0.1–0.1) | 0.2 | (0.1–0.3) | 0.3 | (0.1–0.5) | 1.2 | (1.2–1.2) |

education (70.8%); this is more than twice the rate of Whites overall, and three to four times the rate of Black and Hispanic adults. Lastly, within the Northeast, South Asians are overrepresented in New Jersey with 34.8% residing there.

Table 2 summarizes the prevalence of current and ever cigarette and smokeless tobacco use in New Jersey and in the US Northeast by race/ethnicity and gender. Overall, 16.9% of males in New Jersey report currently smoking cigarettes and South Asians had current smoking rates (12.0%) below their other racial/ethnic counterparts. This pattern is consistent in the Northeast. In general, South Asian females had low rates of cigarette smoking both in New Jersey and the Northeast.

Smokeless tobacco use is predominately a white male behavior in the US, yet in NJ, South Asian males (2.7%) have the highest rates of current use among males. In the northeast, South Asian males (1.4%) currently use smokeless tobacco at a rate somewhat lower, but not significantly different from White males (2.3%). These rates are somewhat confounded by the different ages and educational status of South Asians, as smokeless tobacco use is more common among younger adults and those with lower levels of education. When education and age are adjusted for in a logistic regression, white males (AOR = 5.8, 95% CI = 3.7–9.4) and South Asian males (AOR = 4.0, 95% CI = 1.5–10.6) had significantly higher odds of current smokeless tobacco use relative to non-White males. Rates of smokeless use are extremely low among females of all racial/ethnic groups both in New Jersey and the Northeast. However, ever smokeless use is notable among South Asian females in New Jersey (1.7%) compared to females overall in New Jersey (0.2%).

Tobacco use behavior among South Asians is not homogeneous (see Table 3). Overall, in the Northeast, while Pakistanis make up only 9.6% of all South Asian males, they are overrepresented among current cigarette smokers, but not ever smokers, raising questions about cessation. Indeed, the ever smoking rate among Pakistani males in the northeast is 24.3%, and the current smoking rate is 22.4%, suggesting that few male Pakistani ever smokers have quit. On the other hand, Indian males who make up 70% of all South Asians, comprise 85.9% of the current smokeless tobacco users. Lastly, the data suggest a possible acculturation effect among females. Indeed, while first generation female Americans of South Asian descent comprise 17.6% of South Asian females overall, they are overrepresented among South Asian female cigarette smokers (50.0%).

## 4. Discussion

This study represents one of the only descriptions of tobacco use by South Asians in the United States at the population level. Despite common misperceptions regarding health behaviors and status, South Asians in this study demonstrate important tobacco-use behaviors including lower quit rates, high rates of smokeless tobacco use, and significant heterogeneity regarding these behaviors. Our data support the existing literature demonstrating lower rates of cigarette use in South Asians than other racial/ethnic groups [10, 11]. Similar to the prior data by Choi et al. [21], our study supports the hypothesis that acculturation has a beneficial effect in Asian American men and harmful effects on women and adolescents.

Despite having lower rates of ever and current cigarette smoking than other racial/ethnic groups, it is important to recognize cigarette smoking behavior differed by country of origin among South Asian males, and the data suggest that Pakistani males who have ever smoked cigarettes continue to use tobacco. This may be partially explained by the age distribution of South Asians, who tended to be younger; a group that is less likely to quit than older ever smokers. However, the findings do raise concern that South Asian immigrants who smoke may be less motivated to quit and/or have a more difficult time stopping smoking. This possibility is supported in the, albeit limited, research literature. In the UK, the intention of South Asian males to give up smoking was similar to the general population; however, actual quit rates were much lower, and utilization of cessation services was lower among South Asians [13]. Reasons for this are unclear. Bush et al. [12] suggested that the social acceptability of smoking in Pakistani and Bangladeshi communities may contribute to a lower quit rate while White et al. [22] noted a low level of awareness of the health risks associated with smoking and insufficient use of professional advice/smoking cessation aids among this population.

Smokeless tobacco use is an especially important behavior among certain South Asians, especially males and those from India. While in the US, smokeless tobacco refers to moist snuff or chewing tobacco, the term "smokeless tobacco" is broad and refers to over 30 different types of products including those indigenous smokeless tobacco products that are most frequently used in South Asia, including but not limited to paan, paan masala, zarda, betel quid with tobacco, and gutka [23–25]. Health effects linked to smokeless tobacco use in general include oral cancer, pancreatic cancer, oral diseases such as periodontal diseases, precancerous lesions, and risk factors for cardiovascular diseases, diabetes, reproductive health effects, and overall mortality [25]. Moreover, there is conclusive evidence that betel quid chewed with and without tobacco, tobacco with lime, and other tobacco mixtures specific to South Asian smokeless tobacco products increase the risk of oral cancer [24]. Not surprisingly, data indicated that oral cancer incidence and mortality among people of South Asian descent are almost twice those of global rates [26] and are largely attributed to the use of indigenous tobacco products [27]. Data from the UK, Canada, and California suggest that South Asian immigrants may maintain these higher rates of oral cancer compared to general population [28–31]. Given the growth in this population, future cancer surveillance is clearly warranted.

Finally, the heterogeneity of this sample of South Asians demonstrates that generalization of tobacco surveillance findings can lead to erroneous conclusions. For example, this group of South Asians illustrates high rates of cigarette smoking among Pakistanis while Indians represent most smokeless tobacco users. While studies conducted in the UK and the US have suggested that South Asians in aggregate may have lower rates of smoking than the general population

Table 2: Prevalence* of ever and current cigarette and smokeless tobacco use among adults in New Jersey and the US northeast by gender and race/ethnicity, 2003–2006/7 Tobacco Use Supplement to the Current Population Survey.

| | New Jersey | | | | | | | | Northeast | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Males | | | | Females | | | | Males | | | | Females | | | |
| | Cigarette use | | Smokeless tobacco use | | Cigarette use | | Smokeless tobacco use | | Cigarette use | | Smokeless tobacco use | | Cigarette use | | Smokeless Tobacco use | |
| | % | 95% CI | % | 95% CI | % | 95% CI | % | 95% CI | % | 95% CI | % | 95% CI | % | 95% CI | % | 95% CI |
| *White* | | | | | | | | | | | | | | | | |
| Ever | 45.9 | (43.8–48.0) | 8.7 | (7.5–9.9) | 39.2 | (37.2–41.2) | 0.3 | (0.3–0.9) | 47.4 | (46.5–48.2) | 13.4 | (12.7–14.0) | 40.4 | (39.7–41.1) | 1.1 | (1.0–1.2) |
| Current | 16.1 | (14.4–17.8) | 1.0 | (0.5–1.5) | 13.5 | (12.2–14.8) | 0.1 | (0.0–0.1) | 19.2 | (18.6–19.8) | 2.3 | (2.0–2.6) | 16.7 | (16.2–17.2) | 0.0 | (0.0–0.0) |
| *Black* | | | | | | | | | | | | | | | | |
| Ever | 36.8 | (30.4–43.3) | 4.2 | (1.8–6.7) | 26.5 | (22.0–31.0) | 0.6 | (0.0–1.2) | 35.9 | (33.1–38.8) | 3.6 | (2.5–4.6) | 27.0 | (25.1–29.0) | 0.8 | (0.4–1.1) |
| Current | 20.6 | (14.8–26.3) | 0.3 | (0.0–0.8) | 14.9 | (11.4–18.4) | 0.0 | (0.0–0.0) | 20.5 | (18.0–23.0) | 0.5 | (0.1–0.9) | 15.7 | (14.0–17.4) | 0.2 | (0.1–0.3) |
| *Hispanic* | | | | | | | | | | | | | | | | |
| Ever | 31.7 | (26.5–37.0) | 2.7 | (1.1–4.3) | 17.1 | (13.4–20.7) | 0.4 | (0.0–0.6) | 32.0 | (29.6–34.4) | 2.9 | (2.1–3.6) | 20.6 | (18.9–22.2) | 0.3 | (0.1–0.4) |
| Current | 18.3 | (14.1–22.5) | 0.0 | (0.0–0.0) | 8.9 | (5.8–12.0) | 0.0 | (0.0–0.0) | 18.9 | (16.9–20.8) | 0.5 | (0.1–0.8) | 11.9 | (10.5–13.2) | 0.0 | (0.0–0.0) |
| *Asian* | | | | | | | | | | | | | | | | |
| Ever | 36.7 | (27.8–45.6) | 0.0 | (0.0–0.0) | 3.3 | (0.3–6.2) | 0.0 | (0.0–0.0) | 28.5 | (24.9–32.1) | 2.0 | (0.8–3.1) | 7.9 | (5.7–10.1) | 0.1 | (0.1–0.3) |
| Current | 18.6 | (10.9–26.3) | 0.0 | (0.0–0.0) | 1.3 | (0.0–3.2) | 0.0 | (0.0–0.0) | 15.9 | (12.7–19.1) | 0.4 | (0.1–0.9) | 4.6 | (2.8–6.4) | 0.0 | (0.0–0.0) |
| *South Asian* | | | | | | | | | | | | | | | | |
| Ever | 30.9 | (21.3–40.6) | 2.7 | (0.0–6.0) | 3.6 | (0.0–7.7) | 1.7 | (0.0–2.6) | 22.7 | (17.6–27.7) | 2.5 | (0.8–4.1) | 4.3 | (1.5–7.0) | 0.7 | (0.1–1.5) |
| Current | 12.0 | (5.4–18.6) | 2.7 | (0.0–6.0) | 3.6 | (0.0–7.7) | 0.0 | (0.0–0.0) | 9.5 | (5.7–13.2) | 1.4 | (0.2–2.6) | 2.6 | (0.2–5.0) | 0.0 | (0.0–0.0) |
| *Overall* | | | | | | | | | | | | | | | | |
| Ever | 41.8 | (40.0–43.6) | 6.7 | (5.8–7.6) | 31.9 | (30.2–33.6) | 0.2 | (0.3–0.7) | 43.7 | (42.9–44.4) | 10.8 | (10.3–11.3) | 35.5 | (34.8–36.1) | 0.9 | (0.8–1.0) |
| Current | 16.9 | (15.4–18.4) | 0.8 | (0.4–1.1) | 12.2 | (11.1–13.4) | 0.1 | (0.0–0.1) | 19.0 | (18.4–19.6) | 1.9 | (1.6–2.1) | 15.5 | (15.0–16.0) | 0.0 | (0.0–0.1) |

*Weighted percentages.

Table 3: Country of origin and immigration status among South Asian adults in the US northeast overall and among ever and current cigarette smokers and smokeless tobacco users by gender, 2003–2006/7 Tobacco Use Supplement to the Current Population Survey.

| | Overall | Ever smoker | Current smoker | Ever SLT | Current SLT |
|---|---|---|---|---|---|
| *Males* | | | | | |
| 1st gen American of South Asian descent | 8.4 | 10.6 | 6.0 | 1.3 | 0.0 |
| India | 70.0 | 66.1 | 57.8 | 90.8 | 85.9 |
| Pakistan | 9.6 | 10.7 | 23.4 | 7.9 | 14.1 |
| Bangladesh | 12.0 | 12.5 | 12.9 | 0.0 | 0.0 |
| *Females* | | | | | |
| 1st gen American of South Asian descent | 17.6 | 46.6 | 50.0 | 31.2 | No current use |
| India | 65.6 | 43.9 | 34.4 | 68.8 | |
| Pakistan | 11.1 | 9.5 | 15.5 | 0.0 | |
| Bangladesh | 5.6 | 0.0 | 0.0 | 0.0 | |

[10, 11], other studies have found high rates of smoking in certain subgroups of South Asians, particularly Bangladeshis [12, 13]. Therefore, population level data collection measures that consider South Asians as a single group will likely miss important country of origin differences in tobacco use behavior, and possibly oral cancer rates. This has critical tobacco dependence treatment implications as effective treatments may vary based on the particular tobacco product.

This study has some limitations that bear mentioning. First, we limited our focus to the northeast. While one out of three South Asians reside in the northeast, the extent to which the findings reported here are generalizable to those residing elsewhere is a valid concern. However, we could find no published reports which provided details on the extent to which those in the northeast may differ from their other US counterparts. Second, the number of participants, especially females and those from particular countries of origin are limited. Therefore, conclusions based on these small numbers should be made with caution. However, a strength of this study was our ability to identify and disaggregate South Asians from Asians overall. Our initial analysis (not shown in this paper) indicated that had we analyzed Asians in aggregate, the rates of smokeless tobacco use would have been masked, yielding extremely low prevalence estimates. Third, tobacco control surveillance systems, which are population-based, ask about traditional "Western" tobacco products, such as cigarettes and moist snuff. Subsequently, we suspect that the prevalence data presented here may underestimate tobacco use as indigenous tobacco products used by South Asians, such as bidi cigarettes, as well as gutka, zarda and paan masala, are not addressed on these surveys. Moreover, some of these indigenous smokeless tobacco products have high levels of tobacco-specific nitrosamines (TSNA) and are associated with substantial health risks that may be greater than their Western counterparts [32]. Lastly, methodological limitations in the TUS-CPS with regards to country of origin restrict our ability to further explore important within group variation for cigarette smoking and smokeless tobacco use among other South Asian immigrant populations (e.g., Sri Lanka, Nepal).

Despite these limitations, this study provides important population level data about differential tobacco use and emphasizes the need for further research that disaggregates South Asian populations. Study findings also point to the need to develop, test, and disseminate multiple, targeted tobacco cessation and treatment interventions that take into account important sociocultural differences among South Asian populations as well as differences based on the particular tobacco product used. While only three empirical studies investigating smoking cessation interventions targeting the broader category of Asian Americans have been documented in the literature, the findings suggest that scientifically valid, culturally tailored, and language-specific interventions are effective in reducing tobacco usage among ethnically specific Asian American populations [33]. Lastly, the use of indigenous smokeless tobacco among South Asians deserves attention in the context of the current "harm reduction" debate, where some tobacco control professionals argue that smokers should switch to smokeless tobacco if they cannot quit. This debate is largely focused around "snus" a very low tobacco-specific nitrosamine (TSNA) product with notably lower health risks than cigarettes. The data available regarding indigenous South Asian SLT products are highly varied with the International Agency for Research on Cancer (IARC) finding higher levels of some TSNA (e.g., NNK) in the smokeless products used in India relative to North American and European smokeless tobacco products [24, 25], and since levels of TSNA's are influenced by many factors (e.g., fermentation, processing, other nontobacco carcinogens such as areca nut), these products may be associated with substantially greater health risks than some Western products. For this reason, even though more data are needed describing the health risks and carcinogenic potential of South Asian SLT products that are available in the US, what is clear is that they are certainly not without harm and should not be marketed to the South Asian community as a safe alternative to smoking.

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*Research Article*

# Family History of Cancer and Tobacco Exposure in Index Cases of Pancreatic Ductal Adenocarcinoma

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*Aim*. To examine interaction between history of cancer in first-degree relatives and tobacco smoking in index patients of pancreatic adenocarcinoma. *Methods*. We carried out a case-control involving 113 patients with pancreatic adenocarcinoma and 110 controls over a 12-month period at the Freeman Hospital, Newcastle upon Tyne, UK. They were all administered a detailed tobacco exposure questionnaire and a family history questionnaire. We calculated cumulative tobacco exposure and risk for pancreas cancer. *Results*. Both smokers (OR 3.01 (95% CI 1.73 to 5.24)) and those with a family history of malignancy (OR 1.98 (95% CI 1.15–3.38)) were more likely to develop pancreatic cancer. Having more than one first-degree relative with cancer did not significantly further increase the risk of pancreatic cancer. Amongst pancreatic cancer cases, cumulative tobacco exposure was significantly decreased ($P = .032$) in the group of smokers (current and ex-smokers) who had a family history of malignancy [mean (SD): 30.00 (24.77) pack-years versus 44.69 (28.47) pack-years with no such history]. *Conclusions*. Individuals with a family history of malignancy are at an increased risk of pancreatic cancer. Furthermore, individuals with a family history of malignancy and who smoke appear to require a lesser degree of tobacco exposure for the development of pancreatic cancer.

## 1. Introduction

The major risk factors for pancreatic cancer are increasing age, tobacco smoking (2004; [1]), and family history of the cancer [2]. The strongest avoidable risk factor in sporadic pancreatic cancer development is tobacco smoking. Familial pancreatic cancer occurs at an earlier age, [3] is clustered in families [4, 5] and has the same poor prognosis as its sporadic counterpart. There are other inherited conditions in which pancreatic cancer occurs as part of a syndrome [6], with 5–10% of pancreatic cancer cases being associated with hereditary syndromes [7], of which familial pancreatic cancer is the most common. About 30% of sporadic pancreatic cancers are causally related to smoking. The remainder have been poorly characterised in terms of aetiology. Although a family history of cancer is known to increase the risk of pancreatic cancer, the additional effect of smoking in these patients is unknown.

Analysis of genetic risk of cancer has shown that most nonhereditary, sporadic cancers develop in genetically predisposed individuals. This predisposition is most likely a result of several low penetrant genes rather than single-gene mutations [8, 9]. These low penetrant genes which by themselves have small relative risks, by virtue of being common in the population may have large population, attributable risks [10]. It has been observed from epidemiological studies that the first-degree relatives of sporadic cancer patients have a 2-3-fold higher risk of developing cancer at the same site and this has also been described for pancreatic cancer but in only retrospective studies [10–12]. Familial clustering observed in certain sporadic cancers without obvious Mendelian inheritance suggests that there is a genetic component in addition to environmental factors [13]. This could be explained on the basis that family members with the similar genetic background are exposed to the same environment and that

this leads to the phenotypic manifestation of the disease. The interplay of environmental and genetic factors appears to play a critical role in the development of pancreatic cancer and this has been well described for its familial form [14]. On this background, it is reasonable to suppose that sporadic adenocarcinoma of pancreas, which forms the majority, is due to gene-environment interaction (GEI). These have been poorly characterised and therefore the majority of sporadic pancreatic cancers have been considered to have no identifiable cause and therefore no high-risk groups are identifiable.

Investigation of this gene-environment interaction provides us with an opportunity to not only understand the disease better but also to stratify risks and develop strategies to improve outcome. This interindividual genetic variation modulates risk for malignancy [15] and identification of these genetic differences forms the basis of risk stratification thereby enabling targeted prevention or earlier diagnosis [16, 17]. This is especially pertinent to pancreatic cancer, as it has a particularly poor prognosis and palliation of symptoms is the most common therapy patients receive—mainly because of late diagnosis although there are other biological factors that play a role. Towards this end we have sought to investigate the relationship between these factors (tobacco smoking and a family history of malignancy) by comparing groups of patients with exposure to a known environmental risk factor for pancreatic cancer but with different genetic backgrounds.

## 2. Methods

Patients with pancreatic adenocarcinoma were prospectively identified, as part of an ongoing molecular epidemiological study. They were invited to take part in this research project which was approved by the Local Research Ethics Committee, and the clinical governance guarantor was the Newcastle upon Tyne Hospitals Foundation NHS Trust, Newcastle upon Tyne, UK. Over a period of twelve calendar months between June 2005 and May 2006, consenting individuals were administered a questionnaire which recorded, in a face-to-face interview, life-style factors including tobacco smoking habit, alcohol consumption, and occupation. A detailed family history relating to malignant disease in their first-degree relatives was also obtained directly from the patient. The World Health Organization Monitoring of Cardiovascular risks (MONICA) questionnaire was used to record detailed tobacco exposure. This enabled us to calculate cumulative tobacco exposure in individuals and to arrive at total pack-years of exposure (total pack years of smoking = (number of *cigarettes* smoked per day × number of years smoked)/20(1 pack has 20 cigarettes)). We also collected data on the mode of diagnosis of the adenocarcinoma of pancreas.

First-degree relatives (FDR) were defined as biological parents, siblings, and offspring. Individuals were considered smokers (current and ex) if they had smoked at least 100 cigarettes in their life-time and nonsmokers if they had not smoked this amount. They were considered ex-smokers if they had stopped smoking for a period of one year. Cases and controls were divided into 2 groups on the basis of a positive family history in first-degree relatives (FDR): FDR+, in whom there was history of malignancy (other than dermatological and primary brain malignancies) in first-degree relatives; and FDR−, in whom there was no such history. FDR1 denoted index cases with a single FDR with malignancy; FDR > 1 denoted those with more than one FDR with malignancy.

We report here the interaction between tobacco exposure and a family history of malignancy in this group of patient. Continuous variables were compared by the student *t*-test and ANOVA for parametric variables and the Mann-Whitney *U* test for nonparametric variables. Correlation was tested using the Pearson's chi-square test. Directional measures were employed as necessary. Odds ratios with 95% confidence interval were calculated to quantify relative risk. SPSS version 15.0 (SPSS, Inc., Chicago IL, USA) was the software platform used for computing these tests. All continuous data are reported as mean (SD).

## 3. Results

*3.1. Study Population.* A total of 145 patients were diagnosed with pancreatic cancer in the study period, one of whom was excluded because of a diagnosis of Li-Fraumeni syndrome which is known to predispose to pancreatic cancer. Three further patients declined to enter the study leaving a total of 141 patients who agreed to take part. The mode of diagnosis of pancreatic malignancy was cytological and/or histological evidence of pancreatic ductal adenocarcinoma in 102 patients (72%) and a combination of radiological, biochemical (serially rising CA19-9), and clinical findings in 39 (28%) patients.

The controls numbering 122 were composed of patients who attended the Freeman Hospital, Newcastle upon Tyne, UK for elective hernia repair surgery ($n = 13$), cholecystectomy ($n = 25$), endoscopic treatment of bile duct stones, and/or benign biliary strictures ($n = 9$) and patients attending the anticoagulation clinic (indications included cardiac arrhythmia, prosthetic cardiac valves in-situ, following pulmonary embolism and other nonneoplastic conditions) ($n = 75$). All patients with benign biliary strictures were followed up for a median of 38 months (range 30–54) and are all currently well with no diagnosis of malignancy. Aetiology of these strictures was previous surgery in the vicinity (cholecystectomy, gastrectomy for benign disease) and previous biliary pancreatitis.

*3.2. Cases and Controls.* Of the 141 cancer patients, 113 with reliable family history were included into this study (family history data being unavailable in 21 and incomplete in 7). Of these 113 pancreatic cancer patients, 60 had a family history of a malignancy in first-degree relatives (caFDR+) whilst 53 were caFDR−. The mean (SD) age at diagnosis for pancreatic cancer cases was 65.1 (10.67) years. There was no difference ($P = .35$) in the mean (SD) age between caFDR+ and caFDR− groups (65.93 (8.90) and 62.23 (13.65) years, resp.). The overall gender ratio was 66 : 47 (m : f), (34 : 26 for caFDR+ and 32 : 21 for caFDR−).


Figure 1: Tobacco smoking behaviour in cases and controls.

The controls numbered 122, of which 110 were included due to constraints of reliability or completeness of family history: controls with a positive family history of malignancy (coFDR+) = 40, controls with a negative family history of malignancy (coFDR−) = 70, controls with unavailable family history of malignancy = 5, and controls with incomplete family history of malignancy = 7. Mean (SD) age of controls was 60.07 (14.34) years. There was no significant difference between the ages of coFDR+ and coFDR− groups. The overall gender ratio was 56 : 54 (m : f) (22 : 18 for coFDR+ and 34 : 36 for coFDR−).

Table 1 summarises the demographics, smoking behaviour, and cumulative tobacco consumption, (overall consumption and stratified by FDR status) of our study population (total 223; cases 113 and controls 110).

*3.3. Tobacco Exposure and Risk of Pancreatic Cancer.* There were 80 pancreatic cancer patients who had experienced significant tobacco exposure at some point in their lives; 33 were current smokers and 47 were ex-smokers who had stopped smoking at a mean (SD) of 19.19 (14.48) years prior to diagnosis of adenocarcinoma of pancreas. The mean (SD) cumulative tobacco exposure in these 80 individuals was 36.98 (27.43) pack-years. There were 33 nonsmokers. The mean (SD) cumulative tobacco exposure in all controls who had experienced tobacco exposure ($n = 51$, current smokers = 29 and exsmokers = 22) was 37 (13.20) pack-years and this was significantly lower ($P = .029$) than that in pancreatic cancer cases. There were 59 nonsmokers amongst the control population. There was no significant difference in the number of current smokers between the cases and controls but significant differences were seen in the numbers of past smokers (Table 1 and Figure 1).

The relative risk for an ever smoker (current and ex) for the development of pancreatic cancer is nearly 3 times that of a non-smoker (OR 3.01 (95% CI: 1.73 to 5.24)). There was no significant difference in the mean age between the cases and controls; however, there was a definite early onset of adenocarcinoma of pancreas in current smokers. A consistent early occurrence of adenocarcinoma of pancreas by about 6-7 years was seen amongst current smokers as compared to nonsmokers, which is independent of family history of cancer (Table 2). FDR status did not affect the age of onset of pancreatic cancer in our cohort (data not shown).

*3.4. Family History of Cancer in FDRs Influencing Risk for Pancreatic Cancer.* A history of malignancy in FDR was present in 60 (m : f = 34 : 26) and absent in 53 (m : f = 32 : 21) cases. Amongst controls, the coFDR+ numbered 40 and coFDR− was 70. The relative risk of development of adenocarcinoma of pancreas for cases with a positive history of malignancy in FDR (caFDR+) was nearly twice that of cases with a negative history of malignancy in FDR (caFDR−) individuals (OR 1.98 (95% CI: 1.15–3.38)). This was independent of any further risk conferred by smoking. Of the 60 cases caFDR+, 36 had a single relative with cancer, 17 had 2 relatives, 6 had 3, and one had 4 relatives with cancer. In total, there were 92 malignancies in caFDR+ and 58 in the coFDR+. The different malignancies in these groups are depicted in Figure 2.

*3.5. Interaction between Tobacco Smoking and Family History of Cancer in FDRs in Influencing Risk for Pancreatic Cancer.* Most importantly amongst cases, there was a significantly decreased cumulative tobacco exposure in the caFDR+ group ($P = .016$) as compared to the caFDR− group. The mean (SD) cumulative pack-years of smoking was 30.00 (24.77) in the caFDR+ versus 44.69 (28.77) in the caFDR− group. Mean (SD) cumulative tobacco exposure in coFDR+ was 22.45 (13.18) and that in coFDR− was 17.33 (14.11). This was not statistically different ($P = .171$). There was, however, a significantly greater tobacco exposure amongst caFDR+ than their coFDR+ case counterparts ($P = .00$) (Table 1). The relative risk for adenocarcinoma of pancreas was higher in smokers in both FDR+ (OR 2.85 (95% CI: 1.24 to 6.65)) and FDR− (OR 3.18 (95% CI: 1.48 to 6.82)) groups, but the amount of tobacco exposure lower in the caFDR+.

Next we divided the cases with a family history of cancer in their FDR into 2 groups—caFDR 1 ($n = 36$): one FDR with cancer and caFDR > 1 ($n = 24$): cases with more than 1 FDR with cancer. We did not find a significant difference in the mean (SD) cumulative pack years of tobacco smoking in between these groups (FDR1: 33.70 (29.24), FDR2: 25.07 (16.68); $P = .269$).

## 4. Discussion

Following significant advances in imaging to aid in patient selection for definitive treatment and improvement in surgical technique and perioperative care, prognosis for resectable pancreatic cancer has improved appreciably. Chemotherapy has a significant role to play in selected cases [18]. However, it does appear that further significant improvement in outcome from the illness will be directly related to the ability to detect the disease early and institute prompt management. This will require identification of high-risk groups in whom targeted screening can be employed and early or precursor lesions recognized [19] and this has been demonstrated successfully in familial forms of the disease [20] and has been found to be cost-effective [21].

Table 1: Summary of results.

| | Pancreatic cancer cases | Controls | *P* value |
|---|---|---|---|
| Total | 141 | 122 | |
| Number included into analysis | 113 | 110 | |
| Male | 66 (58%) | 56 (51%) | ns |
| Female | 47 (42%) | 54 (49%) | ns |
| Mean age | 65.1 (10.67) | 60.07 (14.34) | ns (*t*-test) |
| Ever smokers | 80 | 51 | $P = .023$ |
| Non smokers | 33 | 59 | |
| FDR+ | 60 | 40 | $P = .010$ (chi-squared) |
| FDR− | 53 | 70 | |
| FDR+ Mean (SD) cumulative tobacco exposure in pack years | 30.00 (24.77)* | 22.45 (13.18)** | .229 (Mann-Whitney) |
| FDR− Mean (SD) cumulative tobacco exposure in pack years | 44.69 (28.47)* | 17.33 (14.11)** | .003 (Mann-Whitney) |
| Mean (SD) overall cumulative tobacco exposure in pack years | 36.98 (27.43) | 21.19 (22.04) | .008 (Mann-Whitney) |
| | *$P = .016$ (Mann-Whitney) | **$P = .171$ (Mann-Whitney) | |

*Compares cumulative tobacco exposure between FDR+ and FDR− amongst pancreas cancer cases.
**Compares cumulative tobacco exposure between FDR+ and FDR− amongst controls.

Table 2: Age of onset of all cases of adenocarcinoma of pancreas ($n = 113$) by smoking status (Mean (SD) years).

| Pancreas cancer patients grouped based on family history of malignancy status | Smoking status | | | | | |
|---|---|---|---|---|---|---|
| | Current smoker | Current and Ex-smoker | Ex-smoker | Non-smoker | Ex and non-smoker | ANOVA *P* |
| Combined caFDR+ and caFDR− ($n = 113$) | 60.12 (8.18) | | 67.59 (10.10) | 66.36 (12.17) | | |
| | 60.12 (8.18) | | | 66.36 (12.17) | | #.005 |
| | | 64.51 (10.01) | | 66.36 (12.17) | | *.40 |
| | 60.12 (8.18) | | | | 67.08 (10.95) | +.001 |

#Compares age of onset of pancreatic cancer between current and non-smokers
*Compares age of onset of pancreatic cancer between non-smokers and combined group of current and ex-smokers
+Compares age of onset of pancreatic cancer between current smokers and combined group of ex- and non-smokers.

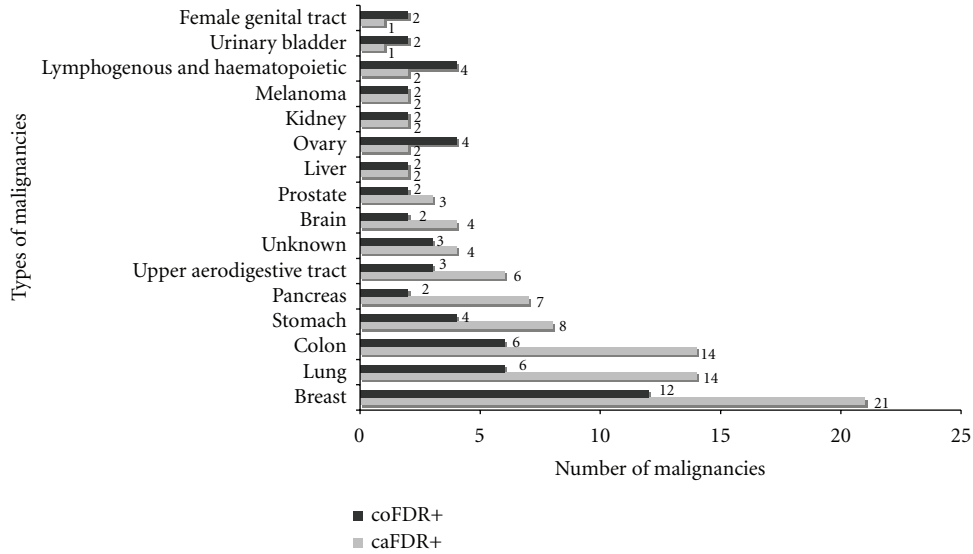


Figure 2: Types of malignancies in caFDR+ ($n = 60$) & coFDR+ ($n = 40$). Total number of malignancies in caFDR+ = 92 (in 60 individuals) and in coFDR+ = 58 (in 40 individuals).

In our prospective hospital-based case-control study, we have seen that pancreatic cancer patients smoked more than our control group and an ever-smoker individual had a 3-times higher risk for the development of pancreatic cancer than a non-smoker. These are well-recognised findings. In addition, however, there were other significant results; smokers on average developed the cancer about 6-7 years earlier than nonsmokers which was independent of a family history of malignancy and has been previously described on the basis of WHO cancer mortality data and SEER cancer incidence data [22]. More importantly a family history of malignancy in first-degree relatives appeared to decrease the amount of tobacco exposure (as measured by pack-years) required for the development of pancreatic cancer. The earlier onset of the disease was however not related to FDR status.

It is accepted that familial pancreatic cancer appears to develop at an earlier age as compared to its sporadic counterpart, and tobacco exposure is the most important factor influencing the penetrance of the FPC gene [14]. Smokers in FPC [23, 24] and in hereditary pancreatic cancer syndromes, specifically hereditary pancreatitis patients [25], develop the disease about 10 years earlier, demonstrating the interaction between an inherited susceptibility to cancer and an environmental carcinogen. A recent report has described gene-environment interaction in a study of cases only, although the sample size was large [26]. We have now shown for the first time that smokers who also have a family history of cancer develop the disease at a lower level of exposure. In smokers, the disease also appears to develop earlier. This might be due to continued or faster accumulation of genotoxic mutations secondary to a variety of factors, one of which might be an inefficient DNA repair mechanism. Other genetic and environmental factors might play a role and this will need further elucidating. For example, a recent report has shown an earlier age of onset of pancreatic cancer in those who had a high BMI during their teen and younger years [27].

The groups of index cases and controls with and without a family history of cancer were comparable given their similar age distribution and gender distribution. We have obtained history of cancer in FDR from index cases and controls and it is known that such information is reliable and accurate especially with regard to FDRs [28]. The reliability of information obtained, however, decreases with regard to other relatives [29, 30], and we have therefore restricted our study to data on first-degree relatives. It has been suggested that, if anything there is under reporting of family history of cancer especially with regard to individuals with colorectal neoplasms [31]. Other details of the illness in the FDR such as age of onset (of the cancer in the relative) are unreliable especially in older probands and we have therefore not utilised such data in our study [29]. We have not performed genetic analysis in this group of patients to confirm that they are not familial cancers as most familial pancreatic cancers are not due to known mutations. It is likely that our patients represent sporadic malignancies due to the fact that the age distribution of the group of patients is normal and there was no difference in the mean (SD) of the age at diagnosis of the index cases in the FDR+ and FDR− groups (65.93 (10.67) and 64.57 (12.38) years).

We have also demonstrated in this prospective group of patients, that those with a family history of cancer as evidenced by the occurrence of a malignancy in an FDR are not only at twice the risk of developing pancreatic cancer (OR 1.98 (95% CI: 1.15–3.38)) but more importantly require less of a genotoxic exposure as compared to those who do not have such a genetic vulnerability (Table 1). Just under 2/3rds of FDR+ index cases ($n = 36$; 59%) had just a single first-degree relative with malignancy. In the FDR+ group, there was a decreased tobacco exposure required for the development of adenocarcinoma of pancreas but this did not depend upon the number of relatives with malignancy, as the FDR > 1 group did not demonstrate a significantly decreased cumulative tobacco exposure. It is well accepted that a family history of cancer is a risk factor for most cancer types. With respect to adenocarcinoma of the pancreas, a recent meta-analysis of seven case-control and two cohort studies involving 6,568 pancreatic adenocarcinoma cases concluded that a family history of adenocarcinoma of the pancreas conferred double the risk (1.80 (95% CI: 1.48–2.12)) for the disease in individuals with such a history compared to those without [32]. A recent cohort study from the PanScan consortium [33] and prospective followup of participants of Cancer Prevention Study-II [34] have suggested an association between family history of various cancers especially prostate cancer and pancreatic cancer. An important additional finding from our study is confirmation that the presence of any malignancy in FDR, apart from dermatological and primary brain malignancies, appears to confer an increased risk for pancreatic adenocarcinoma. We have not performed specific FDR malignancy associated risk analyses in view of the small size of our study population. This is, however, intended for the future when a sufficiently large number of cases have been accrued.

In the presence of a family history of malignancy (i.e., increased susceptibility), a decreased dose of an environmental carcinogen is sufficient to cause cancer (cumulative tobacco exposure in FDR+ (30 (24.77) versus FDR− (44.69 (28.47) ($P = .00$)). It is possible that the decreased tobacco dose demonstrated in the caFDR+ group is due to a genetic or other environmental factor which potentiates the genotoxic effect of tobacco-derived carcinogen by either impairing the processing of tobacco-derived carcinogen into inactive metabolites or causing the inefficient or incomplete repair of genetic damage induced by it. Genetic factors such as poor DNA repair, impaired carcinogen metabolism and environmental factors may interact in the development of tobacco-related cancers, including that of the lung, bladder and head and neck [35–38]. There is some evidence for this in pancreatic carcinogenesis too from molecular epidemiological studies: the presence of XRCC2 Arg188His polymorphism modulates risk for pancreatic cancer amongst smokers [39]; XPD gene polymorphisms—exon 10 Asp(312)Asn and exon 23 Lys(751)Gln polymorphisms—influence risk for smoking associated adenocarcinoma of the pancreas [40]; XRCC1 399Gln allele determines susceptibility to smoking induced pancreatic cancer [41]; deletion polymorphism in

GSTT1 is associated with an increased risk of adenocarcinoma of the pancreas amongst Caucasians [42]. None of these studies, however, has ascertained the risk for smokers carrying these genotypes in the presence of a family history of malignancy. Our findings point to the presence of a high-risk group for adenocarcinoma of the pancreas. This cohort needs further characterisation and replication in larger population based and molecular epidemiological studies.

Identifying risk might help stratify individuals for pancreatic cancer screening but screening is not well established, the pickup rate is low and the false positive rate is relatively high. Surgery usually means a total pancreatectomy with all its potential complications. If we are able, however, to better quantify the risk, the benefits might be greater and identifying genetic and environmental factors is important. With the completion of the human genome project and advances in molecular epidemiological techniques, these low penetrant/polymorphic genes should become more frequently identified and their function understood; for example, genome-wide association studies have identified smokers with a non-O blood group as a significant high risk group for pancreas cancer as compared to nonsmokers of non-O blood group (OR 2.68 (95% CI: 2.03–3.54)) [43, 44]. Similarly identification of high-risk groups such as smokers with a positive family history of cancer could have implications for the earlier diagnosis by making screening for the disease possible leading to the prospect of long-term survival if not cure for more patients.

## 5. Summary

Smoking increases the risk for pancreatic cancer by about 3 times and current smokers develop the disease about 6-7 years earlier than nonsmokers. This risk is irrespective of a family history of any malignancy. In the presence of a family history of any malignancy, regardless of smoking, the risk for pancreatic cancer is double. In individuals with a first-degree family history of malignancy, the development of pancreatic cancer appears to occur at a lower level of cumulative tobacco exposure than in those patients without such a family history.

## Conflict of Interests

The authors declare that they have no conflict of interests.

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## Explanation of Terms and Abbreviations

| | |
|---|---|
| FDR: | First-degree relatives |
| caFDR: | First-degree relatives of cases |
| caFDR+: | First degree relatives of cases with a history of malignancy |
| caFDR−: | First degree relatives of cases without a history of malignancy |
| coFDR: | First degree relatives of controls |
| coFDR+: | First degree relatives of controls with a history of malignancy |
| coFDR−: | First degree relatives of controls without a history of malignancy |
| FDR1: | Index cases with a single FDR with malignancy |
| FDR > 1: | Index cases with more than 1 FDR with malignancy |
| MONICA questionnaire: | Monitoring of Cardio-vascular risks questionnaire |
| CI: | Confidence interval |
| SNP: | Single nucleotide polymorphism |
| CA19-9: | Carbohydrate antigen 19-9 |
| EUS: | Endoscopic ultrasound |
| FNA: | Fine needle aspiration |
| ERCP: | Endoscopic retrograde cholangio-pancreatography |
| Pack-years of cumulative smoking: | = (number of *cigarettes* smoked per day × number of years smoked)/20(1 pack has 20 cigarettes). |

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*Research Article*

# Smoking, Alcohol, and Betel Quid and Oral Cancer: A Prospective Cohort Study

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We aimed to investigate the association between smoking, alcoholic consumption, and betel quid chewing with oral cancer in a prospective manner. All male patients age ≥18 years who visited our clinic received an oral mucosa inspection. Basic data including personal habits were also obtained. A multivariate logistic regression model was utilized to determine relevant risk factors for developing oral cavity cancer. A total of 10,657 participants were enrolled in this study. Abnormal findings were found in 514 participants (4.8%). Three hundred forty-four participants received biopsy, and 230 patients were proven to have oral cancer. The results of multivariate logistic regression found that those who smoked, consumed alcohol, and chewed betel quid on a regular basis were most likely to develop cancer (odds ratio: 46.87, 95% confidence interval: 31.84–69.00). Therefore, habitual cigarette smokers, alcohol consumers, and betel quid chewers have a higher risk of contracting oral cancer and should receive oral screening regularly so potential oral cancer can be detected as early as possible.

## 1. Introduction

Smoking is one of the most important risk factors for developing oral cancers [1, 2]. Oral cancer is currently a major global health issue [3]. In developing countries, oral cavity cancer is estimated to be the third most common malignancy after cancer of the cervix and stomach [4]. Oral cancer has also been one of the top 10 causes of death from cancer since 1991 in Taiwan and the death toll for oral cancer in males has been rising at a surprising rate [5].

No significant advancement in the treatment of oral cancer has been found in recent years. Although better combinations of multidiscipline approach have improved the quality of life in oral cancer patients, the overall 5-year survival rate has not improved much over the past decades [6]. Therefore, primary prevention such as cessation of tobacco smoking and alcohols drinking along with early detection is necessary control procedures to improve the prognosis of oral cancer [7].

Other risk factors have been reported to be closely associated with oral cancers including alcoholic consumption, betel quid chewing [8], poor oral health [9], and human papilloma virus infection [10]. The incidence of oral cancer among patients who had the habit of tobacco smoking was 8.4 fold higher than that among patients who did not [8]. Another study also found the smokers had a 6.41-fold increase in the risk of contracting oral cancer [11]. However, few prospective cohort studies for the risk factors of developing oral cancer have been performed. Therefore, this study aimed to investigate the relationship between smoking, alcoholic consumption, and betel quid chewing and oral cancers in a prospective manner. The synergistic effect of smoking, alcoholic consumption, and betel quid chewing was also examined.

## 2. Materials and Methods

This study was conducted in Taichung Veterans General Hospital, a tertiary refer center in central portion of Taiwan. All male patients who visited our clinic age 18 or older were eligible for enrollment in current study. Those who were

Table 1: Descriptive and bivariate analyses of the studied population.

| Variables | No. of patients (% in column) ($N = 10,657$) | Oral cavity cancer* | | $P$ value |
|---|---|---|---|---|
| | | Yes | No | |
| | | No. of patients (%) | No. of patients (%) | |
| Age | | | | |
| 18–39 years | 2368 (22.2%) | 17 (0.7%) | 2314 (99.3%) | <.001 |
| 40–49 years | 1879 (17.6%) | 61 (3.3%) | 1777 (96.7%) | |
| 50–59 years | 2118 (19.9%) | 94 (4.5%) | 1977 (95.5%) | |
| ≥60 years | 4292 (40.3%) | 58 (1.4%) | 4189 (98.6%) | |
| Habitual smoker | | | | |
| Yes | 2268 (21.3%) | 174 (8.0%) | 1993 (92.0%) | <.001 |
| No | 8389 (78.7%) | 56 (0.7%) | 8264 (99.3%) | |
| Habitual drinker | | | | |
| Yes | 1569 (14.7%) | 138 (9.2%) | 1356 (90.8%) | <.001 |
| No | 9088 (85.3%) | 92 (1.0%) | 8901 (99.0%) | |
| Habitual betel quid chewer | | | | |
| Yes | 758 (7.1%) | 126 (18.3%) | 564 (81.7%) | <.001 |
| No | 9899 (92.9%) | 104 (1.1%) | 9693 (98.9%) | |
| Abnormal mucosa lesion | | | | |
| Yes | 514 (4.8%) | 230 (66.9%) | 114 (33.1%) | <.001 |
| No | 7974 (95.2%) | 0 (0%) | 10143 (100%) | |

* Those with abnormal mucosa lesions but no further biopsy were excluded ($N = 10,487$).

reluctant to join this study were excluded. Participants were first asked to describe their personal habits during the past 6 months, including tobacco use, alcohol consumption, and betel quid chewing. Those who smoked cigarettes, drank alcohol, or chewed betel quid only on special occasions such as wedding banquets, family reunions, or birthday parties were not considered as habitual users. Next, visual inspection of the oral cavity was performed under adequate lighting and with proper instruments. A nonhealing ulcer for more than 2 weeks, a persistent white or red lesion, a lesion that bleeds easily, or an irregular surface lesion inside the oral cavity were regarded as positive findings. Punch biopsy of abnormal lesions was performed after a detailed explanation. If the patient hesitated about further biopsy, followup was strongly recommended.

*2.1. Statistical Analysis.* This study used descriptive statistics for general data presentation. Comparisons of nominal or ordinal variables between the patients proven to have oral cancer and those without oral cancer were analyzed by the Chi-square test. Furthermore, relevant factors for contracting oral cancer were analyzed by a multivariate logistic regression model. All statistics were calculated by SPSS for Windows, version 10.1 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered as $P < .05$.

## 3. Results

A total of 10,657 patients were enrolled in this study from March 2005 to December 2008. All were male, and their ages ranged from 18 to 96 years with an average age of 55.2 years (±18.6 years). Habitual smokers accounted for 21.3% ($N = 2,268$) of the studied population, whereas habitual drinkers and betel quid chewers accounted for 14.7% ($N = 1,569$) and 7.1% ($N = 758$), respectively. Among habitual smokers, 1,068 participants (47.1%) were smokers only, whereas 534 participants (23.5%) were also alcohol consumers, 146 participants (6.4%) had the habit of betel quid chewing additionally, and 520 participants (22.9%) were also alcohol consumers and betel quid chewers. The majority of betel nut chewers (87.9%, $N = 666$) were also smokers and only 6.5% ($N = 49$) were solely betel quid chewers.

Five hundred fourteen participants (4.8%) were recorded to have positive lesions. Among those with positive lesions, 344 participants (66.9%) underwent oral cavity biopsy. Among those who received biopsy, 230 participants (66.9%) were proven to have oral cancer. One hundred seventy participants (23.9%) with abnormal oral lesions were lost to followup, and no further pathological report could be obtained. In order not to confound further analysis, we excluded those who had positive lesions yet no additional biopsy obtained during the follow-up period. Other descriptive statistics are presented in Table 1.

*3.1. Bivariate Analysis.* After dividing all the participants into two groups (those with and those without pathologically proven cancer), the oral cancer group consisted of 230 participants and the control group consisted of 10,257 participants. Comparisons of variables between the two groups are detailed in Table 1.

Table 2: Multivariate logistic regression model of risk factors for developing oral cancer.

| Variables | No. of patients ($N = 10,487$) | Odds ratio | 95% Confidence Interval | | $P$ value |
|---|---|---|---|---|---|
| | | | Lower limit | Upper limit | |
| Age | | | | | |
| 18–39 years† | 2330 | 1.00 | | | <.001 |
| 40–49 years | 1838 | 3.68 | 2.11 | 6.41 | <.001 |
| 50–59 years | 2071 | 6.19 | 3.62 | 10.58 | <.001 |
| ≥60 years | 4247 | 3.66 | 2.09 | 6.43 | <.001 |
| Personal habits | | | | | |
| None† | 7775 | 1.00 | | | <.001 |
| Smoking only | 1040 | 5.13 | 3.17 | 8.32 | <.001 |
| Alcohol consumption only | 464 | 1.33 | 0.48 | 3.74 | .584 |
| Betel quid chewing only | 43 | 11.95 | 3.54 | 40.33 | <.001 |
| Smoking + alcohol | 518 | 9.88 | 6.05 | 16.12 | <.001 |
| Smoking + betel quid | 135 | 26.56 | 14.52 | 48.58 | <.001 |
| Alcohol + betel quid | 38 | 21.84 | 8.04 | 59.36 | <.001 |
| Smoking + alcohol + betel quid chewing | 474 | 46.87 | 31.84 | 69.00 | <.001 |

† Reference group.

There were significant differences between the two groups based on the age ($\chi^2$ value = 100.82, $P < .001$). Besides, there was also a significant difference between the two groups in personal habits such as smoking, alcohol consumption, and betel quid chewing.

*3.2. Logistic Regression Model.* Using the results of pathological examination as a dependent variable, a multivariate logistic regression model for exploring the relevant risk factors for developing oral cancer was created. We found that those aged 50 to 59 years were more likely to contract oral cancer when compared with those less than 40 years old (odds ratio (OR): 6.19, 95% confidence interval (CI): 3.62–10.58, $P < .001$). Furthermore, those who were habitual smokers, alcohol consumers, and betel quid chewers had the highest risk of developing oral cancer when compared with those who did not have these habits (OR: 46.87, 95% CI: 31.84–69.00, $P < .001$). The detailed results are shown in Table 2.

## 4. Discussion

The remarkable increase in the per capita consumption of tobacco and alcohol might be the reason why the incidence of oral cancer increased after 1915 in the United States and other regions around the world [12]. In Taiwan, the annual production of betel nut has also increased year by year since 1981 [13]. This explains why the incidence of oral cancer has rapidly increased in Taiwan and why prevention of oral cancer has become a major public health issue.

Smoking, alcoholic consumption, and betel quid chewing are well-known risk factors associated with oral cavity cancer. However, the estimation of relative risks for contracting oral cavity cancer mostly came from case-control studies [8, 11–13]. In current study, we conducted a prospective cohort study to avoid selective bias that inevitably exits in the case control study.

In this hospital-based study, 10,657 participants received oral cavity inspection and 514 participants (4.8%) were found to have abnormal mucosa lesions. The reported percentage of suspicious lesions in the literature ranges from 1.3% to 16.3% [14]. The differences between the results of this study and other studies may be explained by different studied populations. Among those in this study who were found to have abnormal lesions, 344 participants later received biopsy and 230 patients were proven to have malignancies. Therefore, the positive predictive rate of this study was 68.9%, which is comparable with that of other studies [15].

The prevalence of smoking in this study was 21.3%, which is comparable to that of previous studies. The prevalence of alcohol consumption and betel quid chewing in this study was also similar to that of previous studies conducted in Taiwan [13]. In addition, almost all betel quid chewers were smokers (666 out of 758 patients), which was a finding of previous studies conducted in Taiwan [8, 13]. Therefore, the composition of the population in this hospital-based study is considered to be similar to the general male population in Taiwan.

Tobacco contains N-nitroso compounds, well-known carcinogens, which play a key role in the malignant transformation of oral cancer [16]. Other tobacco carcinogens include the polycyclic aromatic hydrocarbons (PAH) and 4-(methylnitrosoamino)-1-(3-pyridyl)-1 butanone (NNK). They can induce specific mutations, particularly G:T

transversions [17]. Chronic exposure to tobacco carcinogens in the oral mucosa causes genetic changes in the epithelial cells. Cumulative genetic changes lead to genomic instability, development of premalignant lesions, and eventually invasive carcinoma. Tobacco may also induce proliferative activity through activation of the EGFR receptor and its downsteam mechanisms. This activates cyclin D1, leading to greater proliferative activity and higher frequency of mutations, thus rendering the cell more susceptible to permanent genetic changes, that in turn may give rise to genomic instability and invasive carcinoma [18].

According to the annual report by the Taiwan Cancer Registry System, the median age of diagnosis for oral cancer is 51.0 years [5]. Consequently, it is easy to understand why those aged 50–59 years in this study were most likely to develop oral cancer. On the other hand, only 17 out of 2,368 patients (0.7%) under the age of 40 in this study were proven to have oral cancer. Thus, it might be reasonable to start oral mucosa screening of males when they reach the age of 40.

Ko et al. in a case-control study showed that the incidence of oral cancer was 123-fold higher in those who smoked, drank alcohol, and chewed betel quid than in abstainers [8]. However, selection bias inevitably exists in case-control studies. In this study, it was interesting to note that those who drank only alcohol did not have an increased risk of developing oral cancer. A possible explanation might be that we did not collect quantitative data on alcohol consumption. In addition, different types of alcoholic beverages have different effects on the development of oral cancers [11]. Previous studies found evidence of the synergistic effects of smoking, drinking, and betel quid chewing on the risk of developing oral cavity cancer [8, 11]. This might be explained by the fact that betel quid chewers are proportionately heavier smokers, which was also true in the current study. Another study proposed that the alcohol might facilitate the passage of carcinogens through cellular membranes. In addition, alcoholic consumption enhanced liver metabolising activity in both humans and experimental animals and might, therefore, activate carcinogenic substances. Furthermore, alcohol might alter intracellular metabolism of the epithelial cells at the target site [19]. As a result, the oral mucosa was more vulnerable to carcinogens brought by smoking and betel quid chewing.

There were certainly some limitations in this study. First, the external validity of the findings is limited because the study was conducted at a single institution and only included patients visiting our clinic for otolaryngological problems. Second, we did not obtain information regarding quantities of consumption. Consequently, the dose-response relationship of these three risk factors for oral cancer cannot be demonstrated. Lastly, we only recruited male patients. In future studies, it would be useful to compare these data with those obtained from female patients.

## 5. Conclusion

In this prospective cohort study, we found a strong relationship between smoking, alcoholic consumption, and betel quid chewing in oral cancer. Synergistic effects endured patients with all the above habits had an over 40-fold higher risk of developing oral cavity cancer than patients who abstained. Therefore, we recommend those aged ≥40 years who are habitual cigarette smokers, alcohol consumers, and betel quid chewers undergo oral mucosa screening regularly so that potential oral cancer can be identified as early as possible.

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*Review Article*

# Tobacco and the Escalating Global Cancer Burden

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The global burden of cancer is escalating as a result of dramatic increases in the use of tobacco in the developing world. The use of tobacco is linked to the development of a broad variety of cancers, mainly lung cancer, the single most common cancer in the world. Tobacco smoking-attributable deaths extends beyond cancer and include stroke, heart attack and COPD. Widening disparities in cancer-related mortality have shifted towards a more dramatic burden in the developing world. Appropriate interventions must be implemented to reduce tobacco use and prevent global mortality that has escalated to epidemic levels. Tobacco control policies, including public health advertisement campaigns, warning labels, adoption of smoke-free laws, comprehensive bans and tax policies are highly effective measures to control tobacco use. Clinicians and academic institutions have to be actively committed to support tobacco control initiatives. The reduction in cancer related morbidity and mortality should be viewed as a global crisis and definitive results will depend on a multilevel effort to effectively reduce the burden of cancer, particularly in underprivileged regions of the world.

## 1. Introduction

The global burden of cancer is escalating, largely due to dramatic increases in the use of tobacco in less developed nations [1]. Indeed, overall cancer rates appear to be increasing in developing countries, even while they remain generally stable or show small decreases in many industrialized countries [2, 3]. Thus, global changes in tobacco use may eventually produce large disparities in cancer-related mortality rates between the developed and less developed countries of the world [4].

The purpose of this paper is to highlight the trend towards increased tobacco use and the increasing cancer burden in developing countries and suggest steps that might be taken to reverse this alarming trend.

Tobacco was widely used by the Mayans and other Native Americans well before Christopher Columbus introduced it to Europe in 1492. Within 150 years after its introduction to Europe, tobacco use was common throughout the world. Over the centuries, the methods of tobacco usage have changed considerably. In the 18th century, snuff held sway; the 19th century was the age of the cigar; the 20th century saw the rise of the manufactured cigarette and with it a greatly increased number of smokers [5]. Although the worldwide use of tobacco has steadily increased since the 16th century, early public statements showed its disapproval as stated by James I of England in his Counterblaste to Tobacco in 1604: "Smoking is a custom loathsome to the eye, hateful to the nose, harmful to the brain, dangerous to the lungs, and in the black, stinking fume thereof nearest resembling the horrible Stygian smoke of the pit that is bottomless." Thus, even though its health risks have been acknowledged for centuries, tobacco use throughout the world continues to increase.

Most people who use tobacco regularly do so because of their addiction to nicotine, a major component of cigarettes. Although the majority of users express a desire to reduce their use or stop entirely, overcoming the addiction is difficult and may require both pharmacologic and behavioral treatments. Recent research has clarified the addictive nature of nicotine, and it appears to be similar to that of the opiates, cocaine, or other illicit drugs [6, 7].

Environmental factors likely also contribute to the increased use of cigarettes. For many, the behavior of smoking is not simply a matter of addiction, nor one of poor self-image, but also occasionally to underlying mental illness [8].

## 2. Tobacco-Associated Cancers

The association between tobacco and lung cancer was initially demonstrated by Doll and Hill in the 1950s in the UK [9]. Since then, additional case-control studies [10] and prospective cohort studies [11] have all affirmed the association between tobacco and the development of lung cancer. Indeed, lung cancer was rare in the early decades of the 20th century, but with the increase in smoking tobacco, it has become an alarming epidemic.

The tobacco hazard, although clearly linked to the development of lung cancer, also causes an increased risk of several other cancers, notably oral, larynx, pharynx, esophagus, stomach, liver, pancreas, kidney, bladder, uterine cervix cancers, and myeloid leukemia [12].

There is a clear dose-response relationship between cancer risk and tobacco use. A lifetime smoker has a risk 20–30 times greater than of a nonsmoker [13]. More than 4,000 chemicals have been identified in tobacco smoke, and some 60 are known or suspected carcinogens [14]. Each cigarette brings approximately 10 mg of soot, tar, ash, phenols, benzpyrene, hydrogen cyanide, formaldehyde, and radioactive polonium 210 into the lungs of the smokers [5].

## 3. The Global Problem

Worldwide, cancer is responsible for 1 out of every 8 deaths (more than HIV/AIDS, tuberculosis and malaria combined [15]), and tobacco use is responsible for one-third of all cancer-related deaths [16]. The International Agency for Research on Cancer (IARC) estimates that there were approximately 12.7 million new cases of cancer diagnosed in the world in 2008, and 7.6 million deaths attributed to it [17].

Furthermore, tobacco is responsible for 87% of all deaths attributable to lung cancer [18], now the single most common cancer in the world. It is estimated that by 2030 lung cancer will be the sixth most common cause of death in the world, compared with its current ranking of ninth [19].

Tobacco smoking-attributable illness extends beyond cancer and includes stroke, heart attack, and COPD. Indeed, total tobacco-attributable deaths are projected to rise from 5.4 million in 2005 to 6.4 million in 2015 and to 8.3 million in 2030 [19], with an estimated 600,000 deaths attributable to second-hand smoke [20]. These projections are based on models that show a three- to four-decade lag between the rise in smoking prevalence and the increase in smoking-attributable mortality that results from it [21].

Yet, if appropriate measures to control tobacco were implemented, a large proportion of these deaths could be averted. A number of indirect methods to estimate the mortality attributable to tobacco use have been developed; however, limitations related to specific countries and age population groups have been noted in the literature [22–25]. Unless there is widespread cessation of smoking, approximately 450 million deaths will occur as a result of smoking by 2050, and most of these will occur in current smokers [26].

For instance, the global burden of lung cancer has shifted significantly from approximately 31% of cases occurring in developing countries, to now up to 55% occurring in these countries [27]. This makes the widening disparities in cancer-related mortality between developed and developing countries even more tragic. Indeed, the World Health Organization (WHO) estimates that 40% of all cancers diagnosed today could have been prevented, partly by maintaining healthy diet, promoting physical activity, and preventing infections that may cause cancer, but largely through tobacco control [28].

Although the contribution of tobacco use to disease and death is well known, less attention has been given to the ways in which tobacco increases poverty and broadens social inequalities [29]. For example, in Vietnam, the amount spent on cigarettes ($US 416.7 million) is enough to feed 10.6–11.9 million people per year [30]. Furthermore, it has been reported that in China, poor individuals may spend up to 60% of their income on cigarettes, taking away money from food and children education [31]. Serious environmental problems are also associated with tobacco production, which requires the greater use of fertilizers and pesticides and massive deforestation for curing tobacco leaves. One tree is wasted for every three hundred cigarettes produced, and it is estimated that land used for tobacco cultivation worldwide could potentially be used to feed about 10 to 12 million people [32]. As in other agricultural sectors, child labor is prevalent in the tobacco farms, particularly in the poorer areas, where up to 80% of children are missing school and/or undertaking hazardous tasks due to farm work [33].

Currently, smoking imposes a huge economic burden in developed countries, responsible for 15% of the total healthcare costs [34]. Developing countries, with higher population growth rates, are not prepared to cope with such increases in their healthcare expenditures.

## 4. Tobacco Industry

In many industrialized countries, tobacco use appears to be declining, largely due to the diligent efforts of public health officials. In response to these declines, the tobacco industry is now targeting third world markets, not only to expand their markets, but also as a source of less expensive tobacco.

The tobacco industry includes some of the most powerful transnational companies in the world. These companies sell about six trillion cigarettes each year, which accounts for the largest share of manufactured tobacco products, comprising 96% of the total value sales [35]. The industry is highly concentrated within a handful of firms. The global tobacco market, valued at US$ 378 billion, grew by 4.6% in 2007 and by the year 2012 is expected to increase another 23%, reaching US$ 464.4 billion [36]. China is the biggest tobacco market, based on total cigarettes consumed. There are some 350 million smokers in China who consume around 2,200 billion cigarettes a year, or about 41% of the global total. However, the industry in China is state owned. Outside of China, the four largest publicly-listed international tobacco companies account for about 46% of the global market. Although the tobacco companies have experienced declines in profits in industrialized

countries, their overall profits are increasing, driven by world population growth, particularly in Asia. The tobacco companies have reacted to stagnating demand on their traditional markets in basically three ways: consolidation (dominating the business by few but very influential companies), diversification (by producing low- and high-quality cigarettes and geographic diversification), and increasing productivity [37]. Worldwide, even if the prevalence of tobacco use falls, the absolute number of smokers will increase due to the huge population of the developing world [38].

The giant multinational cigarette companies generally find that the political and social climate in the developing world is conducive to their business [39]. Governments in these countries use tobacco taxation as a source of much needed revenue and, therefore, do very little to discourage tobacco use. Furthermore, people in the developing world are generally much less knowledgeable about the health risks associated with cigarettes, and there exist very few anti-smoking campaigns, with tobacco products often carrying no health warnings [40]. In Pakistan, for example, health warnings, even if available, tend to be very vague and poorly understood [41]. High-tar cigarettes, banned in developed countries, continue to be sold in the developing world. For example, nicotine contents for Indonesian kreteks or clove cigarettes are between 1.7 and 2.5 mg per stick compared with <0.05 and 1.4 mg per stick for cigarettes sold in the USA [42].

Yet, the tobacco companies are continuing their marketing efforts in the industrialized countries as well. Although no other consumer product is more dangerous or kills as many people as does tobacco, it still remains the most advertised product in the USA, with estimated advertising expenditures in the tens of billions of US dollars every year [35].

Facing global antitobacco forces, the tobacco industry is already moving beyond what they refer to as "light" and "mild" cigarettes to a new generation of tobacco products referred to as "potential-reduced exposure products" (PREPs) [43]. These products, which have been in development for decades, are the next step after filters and low-delivery "light" and "mild" cigarettes. The essential idea behind PREPs is that they will deliver the levels of nicotine required for a smoker's addiction with less (but some) of the toxins associated with smoking [44]. Yet, these products clearly are associated with alarming health risks, downplayed by the tobacco industry.

In May, 1999, researchers of the World Bank's Health, Nutrition and Population sector published a paper entitled "Curbing the Epidemic: Governments and the Economics of Tobacco Control." This document concluded that tobacco control is not only good for health, but also good for the economy. Yet, multinational tobacco companies have attempted to use their own "economic impact studies" to convince governments that, contrary to the World Bank's conclusion, tobacco use benefits the economy. Thus, the tobacco industry continues its diligent efforts to undermine any threat to its profits. There are several investigators who have argued that the tobacco industry propagates disinformation, manipulates research, and generates faulty information concerning the effects of tobacco use and second-hand smoke [7, 45].

## 5. The Growing Problem in the Developing World

Worldwide, cigarette consumption is increasing at a rate of about 3% annually. In Asia, Southern and Eastern Europe, and developing countries, tobacco use is increasing at about 8% per year. Yet, in some industrialized countries, smoking rates are decreasing at about 1% a year, largely due to the implementation of significant anti-tobacco programs. As with all other epidemics involving a major behavioral component, the exact timing, duration, and magnitude of the smoking epidemic will vary significantly from one country to another. In China and many other developing countries, the rate of tobacco-related deaths is rising rapidly. China is now beginning to face the detrimental consequences of tobacco use, as many millions of individuals who began smoking in adolescence are now aging. Yet, it will be around 2030 before the epidemic of tobacco-related deaths peaks in China at the level achieved in the United States in 1990 [46]. Indeed, lung cancer rates in China have already been increasing about 4.5% a year. These trends reflect significant policy deficiencies towards tobacco use in developing countries.

Most cigarettes are now consumed and produced in Asia. China alone produces close to 40 percent of world total, followed by India, Brazil, and USA (Figure 1). Neither tobacco nor cigarettes are a homogeneous product. Different conditions in the tobacco growing areas, (type of soil, rainfall, irrigation, and climate) handling and processing, ultimately will influence the quality of the leaf and the smoking product. Most manufacturers use a blend of different tobaccos in their product. However, the tobacco's leaf quality and additive contents will affect the particular taste of a cigarette brand and certainly the price [47].

The increasing incidence of cancer in developing countries reflects a transition in the global burden of disease away from one previously dominated by infectious diseases. This shift is also partly due to the ageing of the population and public health interventions such as vaccinations and the provision of clean water and sanitation in the developing world, all of which have served to reduce the burden of infectious diseases.

Also some of the environmental, social, and structural changes linked to the transformation of a country from agrarian to industrial and then to a postindustrial state may lead to increased longevity in the population. As cancer is more common in the older age groups, cancer rates are expected to increase accordingly.

Yet, public health interventions can effectively lower the cancer rates. Low- and middle-income countries, faced with the tobacco epidemic, can learn from the tobacco-control successes in high-income countries by enacting cost-effective tobacco-control policies. Such policies can effectively reduce the burden of cancer.

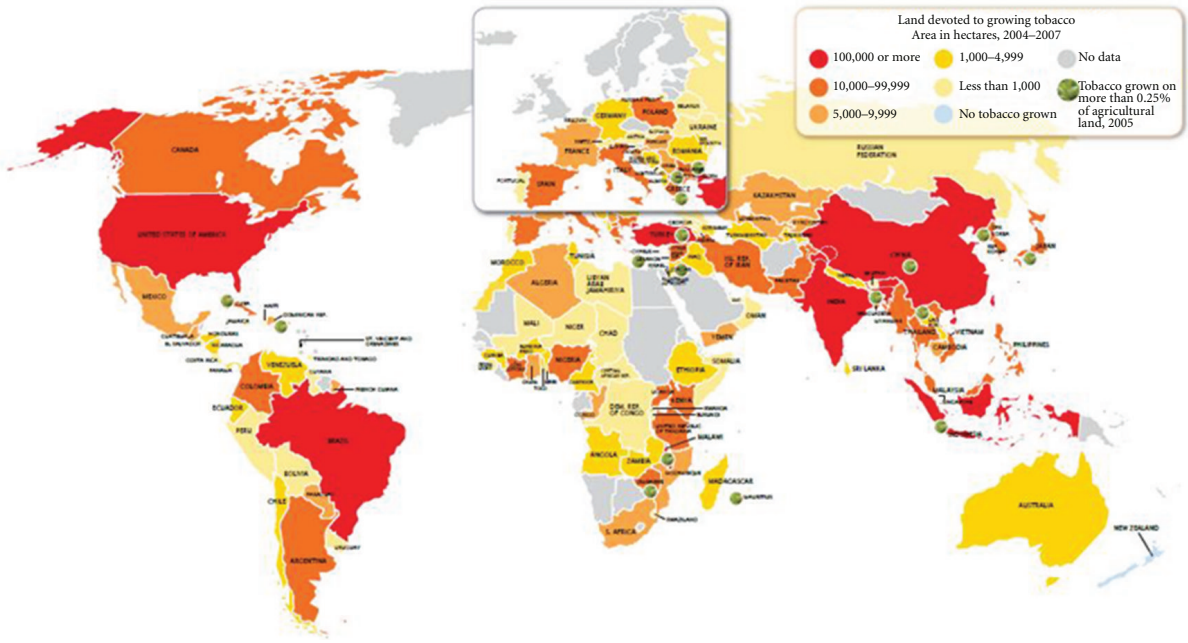


Figure 1: World land devoted to growing tobacco. The Tobacco Atlas, third edition. "Reprinted by the permission of the American Cancer Society, Inc., *The Tobacco Atlas, 3rd Edition.* American Cancer Society 2009, http://www.cancer.org/. All rights reserved."

## 6. Global Approaches to an Escalating Cancer Burden

Many interventions (public health advertising campaigns, warning labels on tobacco products, etc.) that were developed in the industrial world to curb tobacco use should be urgently implemented in the developing world. Interventions to reduce tobacco use may not only avert a large burden of unnecessary deaths, but also save governments huge health care costs. To prevent death or morbidity from cancer, interventions should target behaviors or risk factors that are responsible for tobacco use, and these interventions should be cost effective [48].

The Disease Control Priorities Project (DCPP), a joint effort of the Fogarty International Center of the US National Institutes of Health (NIH), World Health Organization (WHO), and The World Bank, was launched in 2001. This project aims to assist decision makers in developing countries find affordable, effective interventions to improve the health and welfare of their populations [49].

The spirit of international cooperation is exemplified in The Tobacco Control Country Profiles database, a data collection initiative led by the American Cancer Society, the World Health Organization (WHO), and the Centers for Disease Control and Prevention. It represents a worldwide information system to support global tobacco control efforts [50].

The World Health Organization (WHO) has led international strategies to eradicate tobacco use. The WHO Framework Convention on Tobacco Control (FCTC), the first global treaty in response to the tobacco epidemic, adopted in 2003, sets the foundation for price and nonprice population-based control interventions to reduce both demand for and supply of tobacco products and provides a comprehensive direction for tobacco control policy at all levels (Table 1). As of October 2010, 172 countries have ratified the treaty, representing 87.3% of the world's population. Up to a 21% reduction in smoking prevalence can potentially be achieved by implementing important interventions, such as increased taxes on tobacco products, enforcement of smoke-free workplaces, controls on packaging and labeling of tobacco products, and a ban on tobacco advertising, promotion, and sponsorship [51].

The Tobacco Control Program of the WHO was developed in response to the globalization of tobacco use. It is based on the principles of the FCTC, provides data-supported effective measures for tobacco control at all levels, and launches an annual global report summarizing the most current status of the application of those strategies. In 2008, WHO introduced a package of principles under the acronym of MPOWER intended to assist in the country-level implementation of effective measures to reduce the demand for tobacco. Table 2 summarizes those key points.

There are dozens of more national and international nongovernmental organizations which address tobacco control as part of their activities and numerous additional partner organizations that promote tobacco control among their initiatives [35].

Despite significant improvements worldwide in cancer diagnosis and treatment, much still remains to be done [52]. Cancer is a global challenge. Health-oriented resources should be allocated to collect accurate cancer data [53]. In

Table 1: Key policy provisions of the WHO framework convention on tobacco control [8].

| FCTC article no. | Policy |
|---|---|
| 6 | Price and tax measures to reduce demand. |
| 8 | Protection from exposure to tobacco smoke. |
| 9 | Regulation of the contents of tobacco products. |
| 10 | Regulation of tobacco product disclosures. |
| 11 | Controls on packaging and labeling of tobacco products. |
| 12 | Programs of education, communication, training, and public awareness. |
| 13 | Bans on tobacco advertising, promotion, and sponsorship. |
| 14 | Programs to promote and assist tobacco cessation, and prevent and treat tobacco dependence |
| 15 | Elimination of illicit trade in tobacco products. |
| 16 | Measures to prevent the sale and promotion of tobacco to young people. |
| 17 | Provision for support for alternative crops to tobacco. |
| 20 | Provision for an epidemiologic monitoring system. |
| 22 | Cooperation among the parties to promote the transfer of technical and scientific expertise on surveillance and evaluation. |

Table 2: World Health Organization MPOWER key points [1].

| |
|---|
| **M**onitor tobacco use and prevention policies. |
| **P**rotect people from tobacco smoke. |
| **O**ffer help to quit tobacco use. |
| **W**arn about the dangers of tobacco. |
| **E**nforce bans on tobacco advertising, promotion, and sponsorship. |
| **R**aise taxes on tobacco. |

developing countries, cancer registries are perceived as a luxury and rarely provided sufficient resources. International scientific societies have defined standards for cancer data collection, starting with a hospital-based registry which can be the first step towards the formation of a population-based cancer registry. The major aim of a cancer registry is to produce and interpret data to develop country-specific research protocols and cancer control plans [54].

In high-income countries, comprehensive bans on all advertising, promotion, and sponsorship protect people from industry marketing tactics and decrease tobacco consumption by approximately 7%. It has been suggested that these preventive measures might be twice as effective in low and middle-income countries potentially reducing global cancer mortality rates [55, 56]. The Family Smoking Prevention and Tobacco Control Act, a United States federal law that gives the Food and Drug Administration (FDA) the power to regulate the tobacco industry, was signed into law on June 22, 2009 by President Barack Obama. The Tobacco Control Act requires that cigarette packages and advertisements have larger and more visible graphic health warnings (including nine new textual warning statements and color graphics depicting the negative health consequences of smoking) and a prohibition on the manufacture of products that use the terms "light," "low," "mild", and similar descriptors [57].

Tax policies that raise the price of tobacco products are the single most effective approach for reducing demand, since consumption is highly influenced by the extent to which smokers can afford to purchase cigarettes [58]. Price increases are especially effective against the initiation of smoking in youth and motivating addicted smokers to quit [59]. A 10% price increase may cause a 4% drop in tobacco consumption in high-income countries and an 8% drop in low- and middle-income countries, in addition to increasing tobacco tax revenue [60]. Additional price cap regulations (wherein a cap is placed on the pretax cigarette manufacturers' price) limits excess profits for the tobacco industry and increases government revenue [61].

The magnitude of the price increase is one of the most important predictors of an intention to quit/smoke compared with the average cigarette price. However, the availability of alternative (cheaper) cigarette sources may reduce but would not eliminate the impact of higher prices/taxes on the expected intention to stop smoking [62]. Illegally sold cigarettes evade taxes, and indeed, smugglers put cheap cigarettes into the hands of those most vulnerable, the developing countries, where those activities have been rising exponentially. Tobacco has now become the world's most widely smuggled legal substance. The World Health Organization estimates that as many as 25% of all cigarettes sold in the world are smuggled. For the international gangs that organize the traffic, it is even more profitable than drug smuggling [63].

Cessation programs have been shown to provide benefits to certain populations [64]. Cessation programs have a role at all levels of the health workforce, including primary care, health specialists, and smoking cessation specialists.

Adoption of smoke-free laws, included in the article 8 guidelines of the FCTC, has been shown to reduce hospital admission for heart attacks and results in an overall decrease in acute coronary events [65]. Multiple successful examples of countries and cities around the world that have implemented smoke-free laws support the fact that with adequate planning and resources, tobacco-free enforcement protect health and profits the economy [66]. Latin America remains at the forefront of global progress with Colombia, Guatemala, Paraguay, Peru, and Honduras recently added to that growing list [67]. The European Union is proposing a full-scale ban on branded cigarettes, forcing tobacco companies across the continent to sell their products in generic, plain packaging. Worldwide, 25 countries already switched from text to graphic health warnings [68].

International organizations and governments have found certain constraints and barriers to succeed in the war against tobacco: lack of adequate technical and financial resources and capacities for tobacco control; weakness or lack of effective national legislation on tobacco control; lack of public and media awareness of the harmful effects of tobacco use; tactics of the tobacco industry in hindering

effective implementation of already adopted legislation or interference in the development of such legislation; lack of or insufficient political will or intersectoral cooperation in tobacco control [69].

Tobacco control policies implemented in high-income countries may not necessarily have a similar effect in low- and middle-income countries, and public health officials should consider this possibility when planning appropriate interventions [70]. In summary, the definitive results in public health improvement will depend on how aggressive a particular government is on implementing the elements of the WHO's FCTC.

## 7. Clinician and Academic Institution-Based Initiatives

Many of the cancers that pose the greatest threat to developing countries are directly linked to tobacco use. In developed countries, most patients have access to a full range of healthcare resources, including smoking cessation programs, but this is not the case in the developing world. In the developing world, primary care physicians and health workers will need to be more involved in cancer control through health promotion programs that emphasize the hazards of tobacco use and prioritize tobacco cessation. Moreover, many low- and middle-income countries will likely see greater increases in quality-adjusted life years (QALYs) through implementation of smoking cessation interventions, tax policies, bans of promotion and advertisement, and adoption of smoke-free laws, mainly because tobacco-related cancers are preventable, and specialty cancer care is often limited in these countries [15].

The role of the health professionals is critical in tobacco control. At the local level, brief clinical interventions should be implemented based on patient's willingness to quit. Strategies should be implemented to advise patients to quit, to reinforce their decision to quit, and identify those who are at risk for relapse to smoking, providing such individuals with counseling, pharmacotherapy, or both [71].

Unfortunately, in many countries, the prevalence of health professional smokers is similar to that of the general population. To set an example, health professionals should be urged to stop smoking [72]. Thus, the eradication of tobacco should become a priority for not only governments, but also medical schools and physicians.

Multiple studies have shown that there are differences in patient approach, assistance [73], and educational role between smoking versus nonsmoking physicians [74]. Smoking physicians benefit from practical assistance in quitting themselves and providing support to their patients [75]. In 2008, the U.S. Department of Health and Human Services launched a Clinical Practice Guideline, which summarizes the most updated recommendations in clinical treatments for tobacco dependence based on systematic review of evidence-based research that should be implemented by every physician [64]. Along the effective strategies available, clinicians also must be committed to follow a code of professional ethics regarding tobacco. Those should include

(1) physicians not smoking,

(2) make tobacco cessation assistance a routine part of oncology care,

(3) establish all medical facilities to be 100% smoke-free,

(4) teaching physicians should lead their students to never become smokers and train them in the principles of smoking cessation,

(5) reject any involvement of the tobacco industry in financing research, training programs, or treatment services for patients,

Several international societies have trained medical and surgical oncologists as part of their effort to address the burden of cancer in developing countries. Available educational resources for clinicians in developing countries include the European School of Oncology (ESO), the International Campaign for the Establishment and Development of Oncology Centres (ICEDOC), the Global Core Curriculum in Clinical Oncology developed by the European Society for Medical Oncology (ESMO), and the American Society for Clinical Oncology (ASCO), among several others [76, 77].

Unfortunately, some academic institutions have received funds from the tobacco industry to support biomedical research. Universities and researchers must understand the motivation underlying such offers of support. By legitimizing the tobacco industry, universities risk their integrity, values, and public trust [78]. Academic institutions should therefore reject offers of funding from the tobacco industry.

## 8. Conclusion

The reduction of cancer-related morbidity and mortality in developing countries should now become an urgent global priority. Developing countries already have enormous limitations in resources and are unable to cope with an escalating cancer burden. Additionally, an escalating cancer burden in developing countries is not in the best interests of the developed world and should be viewed as a global crisis. Urgent efforts are now needed to curb the widespread use of tobacco and thereby effectively reduce the burden of cancer, particularly in underprivileged regions of the world.

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*Clinical Study*

# The Gap between Tobacco Treatment Guidelines, Health Service Organization, and Clinical Practice in Comprehensive Cancer Centres

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Smoking cessation is necessary to reach a higher quality of life, and, for a cancer patient, it represents an important step in improving the outcome of both prognosis and therapy. Being a cancer patient addicted to nicotine may be a critical situation. We conducted a survey to monitor how many comprehensive cancer centres in Italy have an outpatient smoker clinic and which kinds of resources are available. We also inquired about inpatient services offering psychological and pharmacological support for smoking cessation, reduction, or care of acute nicotine withdrawal symptoms. What we have witnessed is a significant gap between guidelines and services. Oncologists and cancer nurses are overscheduled, with insufficient time to engage in discussion on a problem that they do not consider directly related to cancer treatment. Furthermore, smoking habits and limited training in tobacco dependence and treatment act as an important barrier and lead to the undervaluation of smokers' needs.

## 1. Introduction

"It is a journey, not a fact": this redefinition of smoking cessation by Swartz Woods and Jaen [1] is particularly significant for smokers with a medical diagnosis. In this approach, every exchange between health system and users may have an educative impact, and not only on the patient. Hospitalization is a "teachable moment" [2] as is every encounter with health personnel an opportunity to interfere with the tobacco epidemic, the most preventable of the world's health problems and responsible for killing more people than AIDS, tuberculosis, and malaria put together [3]. In many illnesses, treating tobacco use and dependence becomes part of the patient's care. This is the case for the treatment of tobacco-related diseases, such as in

(a) most cardiovascular treatments [4],
(b) cancer care [5],
(c) respiratory treatments [6],
(d) every surgical treatment [7],
(e) treatment of patients who need inhaled drugs [8],
(f) prevention of immune system impairment [9, 10].

These considerations should lead to the implementation of a sound policy in every health organization, but the gap between theory and clinical practice becomes increasingly obvious.

In the last few years, Italy has made political progress in the field of tobacco control, such as the 2005 law ensuring smoke-free workplaces and hospitality venues. However, 11.1 million (21.7%) adults in Italy are still current smokers [11], and only 375 accredited antismoking centres are operating [12].

Table 1: According to Hughes J. R. Nicotine withdrawal symptoms. Effects of abstinence from tobacco: valid symptoms and time course.

| Withdrawal Symptoms | Peak | Duration |
|---|---|---|
| Anger/irritability/frustration | Within first week | 2, 4 or more weeks |
| Anxiety | 3 day | 2 weeks |
| Dysphoria (depressed mood and negative affect) | 1, 2, 3 weeks | 4 weeks |
| Difficulty concentrating | 2, 3 days | 3, 4 weeks |
| Impatience | | 3, 4, or more weeks |
| Insomnia (sleep fragmentation) | | |
| Restlessness | 1, 3 days | 2, 4 weeks |

Whereas the law is being respected in almost all bars and restaurants, irony wants it that many hospitals are still lagging behind in its implementation. We surveyed the major general hospitals in Milan, the heart of Lombardy's Health System and one of the most developed regions in Italy. Not one of the eight Milan General Hospitals made the NRT (nicotine replacement therapy) available to clinicians to deal with smoking cessation or acute nicotine withdrawal syndrome developed during the inhospital stay. In October 2008, we attended a national Health Promoting Hospitals' Conference, and were impressed by what was stated on the situation of tobacco control in three of the eight Milan General Hospitals. In the first hospital, "the No-Smoking Ban is frequently eluded not only in places like coffee break areas, bathrooms, locker rooms, offices, but also in places frequented by patients like examination rooms, the infirmary and recovery rooms." This study was signed by the Medical and Surveillance Services and by the Quality Control Service. In the second hospital, the study conducted by the Pneumology Rehabilitation Services showed that 45% of nonsmoker employees are exposed to second-hand smoke during working hours. In the third general hospital, another study signed by the Pneumology Medical Board indicated that non-smokers exposed to second-hand smoke in hospitals accounted for 83% for men and 88.4% for women [13].

The cancer "setting" might be a good chance to implement the smoking cessation guidelines, considering that 23.9% of all cancer deaths (33.4% men and 9.6% women) is due to smoking habits [14].

Among hospitalized cancer patients, smokers constitute about 24.5% of the total figure and former smokers about 48.2% [15].

Smoking cessation is an important part of cancer treatment. Different studies indicate that the smoking habit influences the outcome of surgical intervention [16], chemotherapy [17, 18], radiotherapy [19], and biological therapies [20].

Smoking cessation is also part of the treatment in the following conditions: lung cancer [21], breast reconstruction using the free TRAM flap [22], liver transplant [23, 24], colorectal surgery [25], and bone marrow transplant [26].

Evidence-based treatments of smoking habit in oncological patients include nicotine replacement therapies, bupropion, varenicline, and behavioural counselling provided individually, in groups, or by telephone [27–29]. However, clinical practice in Italian cancer departments is still far from such a standard.

In the year 2000, at the National Cancer Institute of Milan, we initiated a smoke-free campaign addressed to patients, visitors, and health personnel. The Institute became a member of the network of smoke-free hospitals, and, since 2003, an antismoking centre has been operating with an average of 350 smokers treated each year. We are at present providing pharmacological and psychological support as an inpatient service to take care of hospitalized smokers. Our pharmacy offers nicotine replacement therapy (NRT), bupropion, and varenicline for smoking cessation and treatment of inpatients' severe acute nicotine withdrawal syndrome. We have summarized, in Table 1, the acute nicotine withdrawal symptoms. Differential diagnosis of this syndrome is made by trained ward clinicians or by neurologists who call upon the intervention of the inpatient antismoking service. In Table 2, we report the last 11 smoker patients diagnosed with this syndrome and treated with NRT. This kind of therapy usually resolves patients' symptoms within 24 hours.

However, our services are merely a drop in the ocean: Italy has 11 million smokers, and a considerable part of the population (approximately 4% or 2.250.000 people, in 2006) lives with a prior cancer diagnosis [30].

We carried out a telephone survey to study the Italian offer of treatment against tobacco use and dependence in 17 cancer centres (CC) belonging to "Alleanza contro il Cancro" (a government initiative that creates a network amongst the most important cancer institutes or cancer departments in general hospitals).

We focussed our survey on the services provided to smoker outpatients (with or without an oncological illness) and on the services provided for inpatients with lung or head and neck cancer. Our first step was to make a telephonic enquiry at the different Institution desks, asking for an outpatient antismoking clinic, and we then contacted the clinic personnel.

In order to favour the cessation or reduction of smoking, six of these clinics offer both psychological and pharmacological support while one only offers group therapy. In Tables 3, 4, and 5, we describe the organization and the resources of the 7 existing outpatient Clinics.

For the second part of the survey, we contacted the head nurses of the 12 lung cancer wards and of the 11 head and neck cancer wards.

NRT, bupropion, and varenicline are available for inpatients in only one cancer clinic (Table 5). Among the existing 12 lung cancer wards only one offers psychological and

Table 2: Characteristics of the last 11 inpatients diagnosed with acute withdrawal symptoms and treated at the National Cancer Institute of Milan.

| Gender | Age | Disease | Setting | Symptoms | Therapy |
|---|---|---|---|---|---|
| M | 56 | Bladder cancer | Surgery | Restlessness | NRT inhaler |
| F | 42 | Breast cancer | Reconstructive | Dysphoria, insomnia | NRT inhaler |
| M | 34 | Metastatic sarcoma | Palliative | Anxiety, restlessness, insomnia | NRT patch |
| F | 74 | Oropharyngeal cancer | Surgery | Restlessness | NRT patch |
| F | 43 | Metastatic ovary cancer | Surgery | Anxiety, insomnia, difficulty concentrating | NRT patch, inhaler |
| M | 66 | Lung cancer | Surgery | Anxiety, restlessness | NRT patch |
| F | 52 | Metastatic breast cancer | Chemotherapy | Insomnia, restlessness, dysphoria | NRT patch, inhaler |
| M | 56 | Oropharyngeal cancer | Surgery | Restlessness, craving | NRT patch |
| M | 28 | Kidney cancer | Palliative | Anxiety, insomnia, craving | NRT patch, inhaler |
| M | 28 | Nose cancer | Palliative | Restlessness, craving | NRT patch, inhaler |
| M | 62 | Colon cancer | Surgery | Anxiety, restlessness, insomnia | NRT patch |

Table 3: Outpatient clinics' treatments for tobacco use and dependence.

| | Yes (%) | No (%) |
|---|---|---|
| NHS-funded antismoking clinic | 5 (29.41) | 12 (70.59) |
| Private antismoking clinic | 2 (11.76) | 15 (88.24) |

Table 4: Outpatient Clinics' multidisciplinary team.

| | Yes (%) | No (%) |
|---|---|---|
| Physician | 6 (85.71) | 1 (14.29) |
| Psychologist | 7 | 0 |
| Nurse | 2 (28.57) | 5 (71.43) |
| Nutritionist | 1 (14.29) | 6 (85.71) |
| Pulmonary physiotherapist | 1 (14.29) | 6 (85.71) |

Table 5: Pharmacological treatments at surgical wards disposal to support inpatient smoking cessation or to care acute nicotine withdrawal syndrome in the 17 cancer centres.

| | Yes (%) | No (%) |
|---|---|---|
| Availability of NRT, bupropion, and/or vareniclina at Hospitals' pharmacy | 1 (5.88) | 16 (94.12) |

Table 6: Treatments of tobacco use and dependence for lung cancer inpatients in the existing 12 surgical wards.

| | Yes (%) | No (%) |
|---|---|---|
| Smoking cessation: pharmacological support | 1 (8.33) | 11 (91.67) |
| Smoking cessation: psychological support | 1 (8.33) | 11 (91.67) |
| Acute nicotine withdrawal syndrome care | 3 (25) | 9 (75) |

pharmacological support for patients motivated or compelled to quit smoking in order to undergo treatment and specific surgery. Acute withdrawal syndrome is usually not detected nor treated and not bedridden cancer inpatients continue to smoke during treatment, including the days prior and following surgery, with the exception of three wards (Table 6). In these cases head nurses, able to make a differential diagnosis, remembered the presence of an acute syndrome among their patients. However, only one ward had a specific inpatient service to deal with such a situation. In one ward, patients have been administered NRT drugs bought by relatives in pharmacies outside the hospital while in another ward patients are treated by anaesthetists without using NRT/bupropion/varenicline.

Table 7: Treatments of tobacco use and dependence for head and neck cancer inpatients in the existing 11 surgical wards.

| | Yes (%) | No (%) |
|---|---|---|
| Pharmacological support | 1 (9.09) | 10 (90.91) |
| Psychological support | 1 (9.09) | 10 (90.91) |
| Acute nicotine withdrawal syndrome care | 2 (18.18) | 9 (81.82) |

We observed the same situation in the existing eleven head and neck cancer wards. Only one ward provides an inpatient tobacco-use treatment service and a comprehensive acute nicotine withdrawal syndrome care. The second ward, listed in the table, treats the syndrome with NRT drugs bought by relatives outside the hospital (Table 7).

## 2. Conclusions

Being a cancer patient addicted to nicotine may be a critical situation. Oncologists and cancer nurses are overscheduled, with insufficient time to engage in discussion on a problem that they do not consider directly related to cancer treatment. Health personnel's smoking habits [31] and limited training in tobacco dependence and treatment act as an important barrier to progress and lead to the undervaluation of smokers' needs.

What must be recognized is that this kind of care can be a great opportunity, not only to support, but to empower smoker cancer patients and to motivate profound changes in their lifestyle. In the cases of acute nicotine withdrawal symptoms, assistance can be of great comfort and help and can further improve relations between patient and hospital operators. It also works towards permanent results in smoking cessation and increases compliance with hospital no-smoking policies [29, 32].

It is of crucial importance that oncologists of every comprehensive cancer centre offer patients, and the whole community, a service of smoking cessation; this service should be connected with the national quit line and with the antismoking centres network. They should also advocate for tobacco-free environments in their patients' communities and share, with their colleagues, their experience in dealing with the tobacco epidemic [33].

The basic needs of smoker cancer patients affect human rights, and recognizing this is an essential part in creating a quality approach to cancer care.

With a nonjudgmental and relationship-centred approach, the aim of the intervention should be to inform and support smoker cancer patients to identify and reach their own health goals according to their own needs and resources.

## Conflict of Interests

The authors declare that there is no conflicts of interest.

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