# Pulmonary Inflammation and KRAS Mutation in Lung Cancer

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**Abstract**

Chronic lung infection and lung cancer are two of the most important pulmonary diseases. Respiratory infection and its associated inflammation have been increasingly investigated for their role in increasing the risk of respiratory diseases including chronic obstructive pulmonary disease (COPD) and lung cancer. Kirsten rat sarcoma viral oncogene (KRAS) is one of the most important regulators of cell proliferation, differentiation, and survival. KRAS mutations are among the most common drivers of cancer. Lung cancer harboring KRAS mutations accounted for ~25% of the incidence but the relationship between KRAS mutation and inflammation remains unclear. In this chapter, we will describe the roles of KRAS mutation in lung cancer and how elevated inflammatory responses may increase KRAS mutation rate and create a vicious cycle of chronic inflammation and KRAS mutation that likely results in persistent potentiation for KRAS-associated lung tumorigenesis. We will discuss in this chapter regarding the studies of KRAS gene mutations in specimens from lung cancer patients and in animal models for investigating the role of inflammation in increasing the risk of lung tumorigenesis driven primarily by oncogenic KRAS.

**Keywords**

Inflammation · KRAS mutation · Tumor microenvironment · COPD · Lung cancer

## Abbreviations

| | |
|---|---|
| AKT | protein kinase B |
| ALK | anaplastic lymphoma receptor tyrosine kinase genes |
| BALF | bronchoalveolar lavage fluid |
| BHT | butylated hydroxytoluene |
| CCSP | club cell secretory protein, aka CC10 |
| COPD | chronic obstructive pulmonary disease |
| COX | cyclooxygenase |
| CXCL5 | C-X-C motif chemokine 5 |
| EGFR | epidermal growth factor receptor |
| ERK | extracellular signal-regulated kinase |
| FOXP3 | forkhead box P3 |
| G-CSF | granulocyte colony-stimulating factor |
| GDP | guanosine diphosphate |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |

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| | |
|---|---|
| GTP | guanosine triphosphate |
| HIF-1α | Hypoxia-inducible factor 1-alpha, aka HIF-1-alpha |
| ICB | immune checkpoint blockade |
| IDO1 | indoleamine 2,3-dioxygenase |
| IFN | interferon-γ |
| IL | Interleukin |
| KC | keratinocyte chemoattractant |
| KRAS | Kirsten rat sarcoma viral oncogene |
| LAG3 | lymphocyte-activation gene 3 |
| MAPK | mitogen-activated protein kinase |
| MCA | 3-methylcholanthrene, aka 3-MC |
| MCP-1 | monocyte chemotactic protein 1 |
| MDSC | myeloid-derived suppressor cell |
| MHC | major histocompatibility complex |
| MIP-1α | macrophage inflammatory protein 1 alpha |
| MIP-2 | macrophage inflammatory protein 2 |
| mTOR | mammalian target of rapamycin |
| NDMA | N-nitrosodimethylamine |
| NNK | nitrosamine4-(methylnitrosamino)-1-(3- pyridyl)-l-butanone |
| NSCLC | non-small cell lung cancer |
| NTHi | nontypeable *Haemophilus influenzae* |
| PAH | polycyclic aromatic hydrocarbons |
| PD-1 | programmed cell death protein 1 |
| PI3K | phosphatidylinositol-3 kinase |
| PTEN | phosphatase and tensin homologue deleted from chromosome 10 |
| ROS | reactive oxygen species |
| SCLC | small cell lung carcinoma |
| TGF-β | transforming growth factor beta |
| TNF | tumor necrosis factor |
| TNM | tumor (T), node (N), metastasis (M) |
| Treg | regulatory T cell |

## 5.1 Lung Cancer Overview

Lung cancer is the leading cause of cancer mortality in the United States with estimated 228,150 newly diagnosed cases and 142,670 deaths in 2019 [1]. Epidemiological data strongly associate exposure to exogenous factors, chiefly from tobacco smoking, to the increased risk of lung cancer [2–5]. Public education to promote abstinence from tobacco smoking and smoking cessation has gained some momentum in the United States, although tobacco use has continued. Therefore, since lung cancer, like many other cancers, takes many years to develop, smokers and ex-smokers still represent individuals with a high risk of developing lung cancer in the years to come [6, 7].

Lung cancer is a heterogeneous disease that comprises multiple histologic subtypes and mainly includes adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell lung carcinoma (SCLC). The first three subtypes are termed collectively non-small cell lung carcinoma and have different clinical features from SCLC. About 85% of lung cancer is non-small cell lung cancer (NSCLC), of which lung adenocarcinoma and lung squamous cell carcinoma are the most common histological subtypes [8, 9]. Although tobacco smoking is the most common etiology for lung cancer and accounts for most lung cancer-related deaths [2–5], environmental and occupational exposure to agents such as arsenic, chromium, asbestos, nickel, cadmium, beryllium, silica, diesel fumes, and coal-burning smoke are also known to cause lung cancer [10–12]. In addition, other possible risk factors include acquired lung diseases, infections, family history of lung cancer, hormonal and reproductive factors, and radon gas seems to also increase lung cancer [3]. Regardless of the identification of well-established causal risk factors, cigarette smoking remains the primary risk factor of the global epidemic of lung cancer.

An extensive effort has been made for lung cancer in regard to screening, minimally invasive techniques for diagnosis, and advancement in therapeutics. However, the 5-year survival rate remains low at only 18% [1], as the majority of patients are diagnosed with locally advanced or metastatic disease, in which the curative surgery is no longer feasible [13]. Regardless of curative surgery for early-stage lung cancer, 20–40% of stage I patients will have tumor recurrence, which remains the main cause of cancer-related death [14–17]. Patients with stage I lung adenocarcinoma, which is the most common histological

subtype, vary in survival outcome. It indicates that the current tumor (T), node (N), metastasis (M) staging system fails to distinguish patients with a higher risk of recurrence for stage I disease following surgical resection [18].

Adjuvant chemotherapy has been shown to decrease disease recurrence and prolonged overall survival in patients with stage II-III disease [19–22], but its role in stage I remains controversial and lacks biomarkers for the indication of treatments. In addition, most patients with advanced or metastatic disease are typically treated with cytotoxic chemotherapy with a modest increase in survival. During the last two decades, the discovery of small molecular inhibitors targeting genetic alternations has improved the survival rates for the subsets of cancer patients. Patients with the mutated epidermal growth factor receptor (EGFR) responded to erlotinib or gefitinib, and those with altered anaplastic lymphoma receptor tyrosine kinase genes (ALK) responded to crizotinib [23, 24]. A study showed that the frequency of *EGFR* and *ALK* mutation in lung adenocarcinoma is 27% and < 8%, respectively, although the frequencies vary by region and ethnicity and the majority of lung cancer patients do not contain these genetic alternations [25]. Even though the subsets of patients with these mutations are treated with targeted therapies, they eventually developed resistance within 1–2 years of starting therapy [26]. Immunotherapy such as immune checkpoint blockade (ICB) has been used recently for lung cancer treatment with promising clinical responses, but the response rate is low and only a small subset of patients benefited from the treatment [27] while most patients who responded to initial ICB treatment finally developed resistance. Several mechanisms for acquired resistance to ICBs have been identified including the defects in interferon-γ (IFN) signaling or major histocompatibility complex (MHC) presentation, and the increased levels of the enzyme indoleamine 2,3-dioxygenase (IDO1), which impaired T cell function by the deprivation of tryptophan [28–30].

Overall, major challenges still remain in lung cancer detection and treatment. Extensive efforts have been made during the past decades to better understand the molecular etiology of the initiation and progression of lung tumors and factors that affect the risk of lung tumorigenesis.

## 5.2 Pathogenesis of Lung Cancer

The development of carcinoma of the lung follows a latent period that spans several decades as the normal respiratory epithelium is exposed to various carcinogens. The response of the normal mucosa to these stresses is believed to be a predictable progression from high-grade dysplasia to carcinoma in situ and eventually resulting in invasive carcinoma [31, 32]. There is an average period of 4–5 years during which time individuals exfoliate markedly atypical cells (that actually represent carcinoma in situ) into the bronchial secretions before the progression to an invasive carcinoma [33, 34].

The progression from normal to initiated cells to invasive tumor is a long and multiple stage process which takes multiple years and proceeds presumably through a series of molecular events leading to an accumulation of genetic variation including mutational, chromosomal, and epigenetic changes [35–39]. In this paradigm, one major pathway to malignant transformation involves structural alterations of cancer-related genes. These genes have been divided into two categories based on whether the gene function is gained or lost. The first involves activated growth-promoting genes (oncogenes), and the second involves inactivated genes that are normally responsible for growth control in the cell (tumor suppressor genes) [40–42].

A large number of oncogenes have been identified, but those that play the most prominent role in cancers are the closely related H-, K-, and N-*RAS* genes [43, 44]. These genes encode for closely similar monomeric 21-kd guanosine nucleotide-binding proteins (RAS) with a weak intrinsic GTPase activity [45–50]. They are related to the G proteins that bind guanine nucleotides with high affinity and are located at the inner surface of the cell membrane, and they play an important role in signal transduction pathways

[47, 50]. The wild type KRAS, once activated by external stimuli, switches from an inactive guanosine diphosphate (GDP)-bound to an active guanosine triphosphate (GTP)-bound conformation. The *RAS* activation switch is catalyzed by the guanine nucleotide exchange factor SOS1 that displaces the GDP, allowing the protein to bind to a GTP. This GTP-bound RAS activates multiple downstream effectors, including those involved the RAF-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK, and the phosphatidylinositol-3 kinase (PI3K)/−protein kinase B (Akt)/mTOR signaling cascades, and thus regulates cell growth/differentiation and apoptosis, respectively. To terminate the downstream signaling, the active RAS protein catalyzes the hydrolysis of the GTP to GDP through its intrinsic GTPase and returns to its inactive state. The catalytic reaction can be increased by the binding of the RAS protein to a GTPase-activating protein, P120GAP, enhancing the GTPase activity and, thereby, accelerating the conversion of GTP-bound to the GDP-bound ras conformation [49, 51–54].

Activation of the *RAS* genes occurs with specific point mutations at only a very few codons, including codon 12, 13, or 61. In wild type *RAS*, amino acids at codons 12, 13, and 61 are in direct contact with the phosphoryl group of GTP and are involved in the catalytic reaction of GTPase. A mutation occurring at any of these codons will induce structural changes within the *RAS* protein, resulting in a reduction or loss of GTPase activity and an activated mutant *RAS*. As a consequence, in cells with a missense mutation at codon 12, 13, or 61 of the *RAS* gene, the mutant *RAS* will remain in its active GTP-bounded state, and constitutively will activate its downstream signaling pathways, thereby increasing abnormal cell growth and differentiation and the risk of tumorigenesis [52, 53, 55]. However, this concept has been questioned because the type of mutation occurring at these codons may affect the ability of guanosine triphosphate to bind to mutant *RAS* [52, 53, 56].

The frequencies and types of mutated *RAS* genes have been found to vary among tumors, depending on the tissue of origin. For instance, KRAS gene was the most frequently mutated in lung, colon, and pancreas tumors [57, 58].

## 5.3 KRAS Mutations in Lung Tumors

There have been extensive studies of KRAS mutations in lung tumors and in other specimens from lung cancer patients, most of them were smokers, from the United States and other parts of the world. These studies showed that KRAS mutations were identified more frequently in the adenocarcinoma subtype (15–30%) and less so in other histological phenotypes, including squamous lung tumors (3–5%) [59–64]. The data also showed that gender did not affect the incidence of KRAS mutations in the lung adenocarcinomas from smokers. For comparison, nonsmoking lung cancer patients are mostly women, while their lung tumors are mostly of the adenocarcinoma subtype that less frequently harbored KRAS mutations (5–15%), compared with smokers. These data suggest that KRAS mutations are primarily associated with exposure to tobacco smoke. The reasons for these varied KRAS mutation frequencies in lung adenocarcinomas are unclear, although differences in sample size, methods used for DNA preparation and mutation detection, and geographical differences may play a role.

The mutations in lung tumors from smokers consisted predominantly of a G to T transversion (~60%), whereas a G to A transition accounted for ~30%. A transition is the conversion of purine (A, G) to another purine base or pyrimidine (C, T) to another pyrimidine base whereas transversion is the conversion of a purine into a pyrimidine or vice versa. The consistent predominance of a G to T transversion in the KRAS gene in lung tumors has been reported in several studies [60, 65, 66] and is characteristic of lung tumors, in contrast to other cancer types, such as colorectal carcinomas, in which the G to A transition predominated [67, 68]. The predominance of G to T transversion, along with the prevalence of the KRAS mutation in the smoking population, sug-

gests that carcinogens in cigarette smoke, in particular the polycyclic aromatic hydrocarbons, (PAHs) may cause these mutations. For instance, benzo(a)pyrene is a known carcinogen that forms adducts with deoxyguanine residues in DNA and to induce mostly G to T transversion in several in vitro systems [69, 70]. The varying incidence of G to A transitions found in the different studies reflects differing carcinogenic exposure, such as exposure to radon or nitrosamines, that can cause this type of mutation. In addition, nicotine can be activated to the nitrosamine 4-(methylnitrosamino)-1-(3- pyridyl)-l-butanone (NNK), which could contribute to G to A transitions observed in some smokers. In mice, NNK, one major tobacco smoke carcinogen, caused mutations in the KRAS gene of lung adenocarcinomas that were almost exclusively G to A transitions in codon 12 [71].

Compared with smokers, the KRAS mutations in lung tumors from nonsmokers consisted mainly of G to A transition [64, 72], suggesting different mutagen origins and/or mechanisms of tumorigenesis in nonsmokers who were mostly women. Nevertheless, most lung cancer is caused by exposure to smoke carcinogens from tobacco and/or from other sources. For instance, there was a high incidence of lung tumors among female nonsmokers in Xuan Wei County (XWC), Yunnan Province, China. These women were exposed to smoky coal emissions for generations, and their lung cancer rate was 5-fold and, in some communes, up to 24-fold greater than the Chinese national average. Investigation of KRAS mutations revealed that their lung tumors carried KRAS mutations, consisting highly of G to T transversion at codon 12 [66] (87–100%). Household fuel surveys indicate that lung cancer was highly correlated with the use of generations of "smoky coal" for domestic combustion [73, 74]. Smoky coal is a low-sulfur (0.2%) medium-volatile bituminous coal used for cooking and heating in XWC homes without chimneys. Characterization of the indoor air from homes using smoky coal showed that XWC residents were exposed to high concentrations of submicron particles that contain mostly organic matter, including large amounts of mutagenic/carcinogenic PAHs [75–77]. These results point to a strong etiologic link between exposure to smoky coal combustion and the high rate of lung cancer harboring KRAS mutations in women living in XWC.

## 5.4 KRAS Mutation Type and Status in the Prognosis of Lung Cancer

In spite of being studied for many years for their role in lung tumorigenesis, only recently have an increasing number of studies shown evidence of prognostic and predictive values of KRAS mutations in lung cancer, although the results were not consistent across studies. For instance, some studies showed that lung tumors with KRAS mutations were more likely to be resistant to therapies [78–82] and to engraft in immunodeficient mice and predict disease recurrence [83] than those without these mutations. Furthermore, it has been suggested that the different KRAS mutation types could lead to different oncogenic KRAS variants.

In lung tumors, KRAS mutations were found primarily at codon 12 (~93%), where cysteine, valine, and aspartate accounted for about 80% of the amino acid changes that substituted for the wild type glycine [60, 84]. These KRAS variants could possess distinct biologic manifestations, including their signaling pathways, transforming potential, and treatment outcomes. For instance, patients with tumors harboring a substitution of codon 12 of arginine, cysteine, aspartate, or valine had a poorer outcome than those whose tumors contained wild type or other amino acids. However, the results were not always consistent among studies, likely reflecting on the small numbers of the relevant amino acid substitutions and patient populations involved in these studies [60, 84].

On one hand, several other studies showed that patients with some types of KRAS mutations had significantly poorer survival, compared with patients with KRAS wild type. In particular, mutant cysteine substitution at codon 12 was associated with poor prognosis, compared with

other mutant KRAS or wild-type KRAS [79–82].

On the other hand, other studies showed there were no differences in prognostic value based on the type of KRAS amino acid substitution present, or the mutant KRAS variants versus wild type KRAS [85, 86]. These discrepancies across studies may reflect from a heterogeneity regarding the stages, the histology, and the treatment modalities of lung cancer. Furthermore, the different methods used for DNA preparation and mutation analysis could also explain the varying results from the different studies.

## 5.5 Mutant KRAS Signaling in Lung Tumorigenesis

Studies have shown that human lung tumors with activating KRAS mutation have higher levels of inflammation, compared with lung tumors without these mutations [87]. This indicates a link between activating KRAS and tumor-associated inflammation. In lung cancer, it has been suggested that KRAS mutation is important in the initiation but may not be sufficient for an effective and complete development of lung adenocarcinoma [88–94]. Lung tumorigenesis initiated by oncogenic KRAS may be further promoted or inhibited by genetic/epigenetic events that activate or suppress other signaling pathways. Factors capable of controlling such events and pathways may impact the development of an initiated cell into a malignant tumor and, therefore, lung tumor incidence.

Several mouse models have been developed to investigate the molecular pathways to lung tumors driven by mutant K-ras [87–94] (mouse homolog of human KRAS). One of such studies used a cohort of conditional mutant mice in which the aspartate substitution at codon 12 of allele of K-ras (K-ras$^{G12D}$) was expressed specifically in mouse CC10-positive bronchiolar epithelial cells [87]. The activation of oncogenic K-ras$^{G12D}$ in these cells led to the development of lung adenocarcinoma. These cells produced inflammatory chemokines, characterized by the production of Macrophage Inflammatory Protein-2 (MIP-2), C-X-C motif chemokine 5 (CXCL5, LIX), and keratinocyte chemoattractant (KC) by cell lines established from the mouse lung tumors, and by the increase of these chemokines in the mouse bronchoalveolar lavage fluid (BALF). These chemicals attracted neutrophils and macrophages within the mouse lung, generating lung inflammation and a pro-tumorigenic environment within the lung.

Other studies showed that mutant KRAS cooperates with alterations of other genes, including the loss of tumor suppressor gene phosphatase and tensin homologue deleted from chromosome 10 (PTEN), one of the components regulating the PI3K/Akt pathway [80–82, 93, 95, 96]. For instance, human NSCLC cell lines that express no detectable PTEN frequently had KRAS mutations, suggesting that alteration in both genes confers a selective advantage in these cells [93]. Another study used mouse models of CCSP-driven expression of oncogenic *K-ras* (Pten$^{\Delta 5/\Delta 5}$; Kras$^{Lox/+}$; CCSP$^{Cre/+}$), in which conditional oncogenic K-ras and Pten null alleles can be targeted specifically in the CCSP-expressing bronchial epithelium. It was demonstrated that, by itself, Pten inactivation had no discernible effect, but it accelerated lung tumorigenesis initiated by oncogenic K-ras. The tumor microenvironment in these mice was enriched in endothelial cells and inflammatory cells and that the lungs expressed high levels of chemokines and growth factors [93]. It has been shown that the interaction between Pten loss and K-ras mutant alters PI3K pathway regulation, enhances the activation of the nuclear transcription factor NF-κB [95–97], up-regulation of downstream cytokines, and creates an inflammatory environment within the lung.

NF-κB plays an important role in the regulation of the expression of genes involved in inflammation, immune responses, cell cycle, apoptosis, and angiogenesis in a variety of cells, including epithelial cells, and deregulated NF-κB plays an important role in tumorigenesis [98–104]. Increased NF-κB activity correlates with expression of oncogenic KRAS and that the p65/RelA subunit of NF-κB is an important oncogenic KRAS effecter in lung cancer [105–107]. For

instance, in mouse studies, activation of the NF-κB pathway has been detected during KRAS oncogene-driven lung adenocarcinoma [105, 108]. Inhibition of NF-κB signaling in the airway epithelium significantly reduces the formation of lung tumors [100], while NF-κB activation in the lungs markedly increases tumor formation [109, 110]. This supports the concept that activation of NF-κB pathway plays an important role in lung carcinogenesis.

## 5.6 Extrinsic Inflammation Promotes Mutant KRAS-Initiated Lung Tumorigenesis

Inflammation is an essential process for host immune responses to prevent pathogen invasion and also involves in wound healing. However, persistent and uncontrolled inflammatory responses are associated with active recruitment of inflammatory cells and the production of mediators such as cytokines, chemokines, growth factors, and matrix-degrading enzymes leading to inflammatory microenvironment [111]. It has been reported that "smoldering" inflammation in the tumor microenvironment has many tumor-promoting effects such as tumor-cell migration, invasion, metastasis, epithelial-mesenchymal transition, and angiogenesis [112]. In addition, chronic inflammation also induces immunosuppressive mechanism associated with accumulation of suppressive cells like myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) as well as the increased immunosuppressive mediators such as Interleukin 10 (IL-10) and transforming growth factor-beta (TGF-β), which help tumors escape from immune surveillance [113, 114]. Although the exact mechanisms of inflammation in promoting lung cancer remain unclear, two hypotheses proposed that an intrinsic pathway driven by genetic alternations leads to neoplasia and inflammation, and an extrinsic pathway driven by inflammatory conditions leads to increased cancer risk [112]. Several mouse models have been applied to explore the effects of extrinsic pulmonary inflammation induced by several agents on tobacco smoke carcinogen-mediated lung tumorigenesis.

Witschi et al. [115] evaluated the effects of the food additive phenolic antioxidant, butylated hydroxytoluene (BHT), on the development of lung tumor in male Swiss-Webster mice following exposure to the tobacco smoke carcinogen urethane. The development of lung tumors was enhanced by a single injection of urethane and chronic exposure to BHT. BHT acted as a promoting agent, as it effectively enhanced tumor formation in mice exposed to BHT after being injected with urethane, but not if they are treated with BHT before urethane injection. This suggested that this mouse treatment system provides an example of two-stage carcinogenesis consisting of initiation by a carcinogen and promotion by BHT [116].

Another study examined the effects of chronic BHT exposure following a single injection of 3-methylcholanthrene (MCA) into BALB/c mice. It was found that treatment with low doses of MCA in this strain does not induce lung tumors, unless BHT exposure follows MCA treatment. BHT administration promotes a 3-fold increase in urethane-induced lung tumor multiplicity [116, 117]. However, BHT administration promotes lung tumor formation only in BALB/c mice but not in CXB4 mice [118]. The MCA/BHT protocol in BALB/c mice thus offers an experimental model for determining the biochemical and cellular nature of how BHT stimulates the selective clonal expansion of initiated cells [117].

Administration of BHT to BALB/c and A/J mice at doses higher than 150 mg/kg caused infiltration of inflammatory cells into the alveoli [119, 120], followed by the reversible pneumotoxicity to mice. In addition, Bauer et al. [118] showed that BALB/c mice treated with BHT developed strong inflammatory responses characterized by transudation of proteins from the blood into the BALF, and an influx of macrophages and lymphocytes into the airspaces. There were also elevated pulmonary concentrations of cyclooxygenase-1 (COX-1) and COX-2 and increased prostaglandin synthesis [118]. For comparison, the CXB4 mice that did not show

any increase in lung tumor formation following treatment with the MCA/BHT protocol were found to be resistant to all of the BHT-mediated increases in inflammatory parameters that occurred in BALB/c mice [118].

Matzinger et al. [121] evaluated the two-stage model of lung tumorigenesis in A/J mice treated with the tobacco smoke carcinogen NNK. They demonstrated that BHT promotes an increased multiplicity of the mouse lung tumors, following NNK exposure. Furthermore, there were some differences in the K-ras mutation patterns identified in the lung tumors. While all mutations in the non-BHT-treated mice consisted of G to A transition occurring at the second base of K-ras gene codon 12, only about one-third of the mutations found in the BHT-treated mice were of this type. This suggests that the NNK-initiated lung tumorigenesis in these mice was altered by BHT-tumor promotion, from oncogenic K-ras-driven pathway to a non-K-ras mechanism.

Wang and Witschi [122] compared the promoting effects of BHT on lung tumorigenesis initiated by urethane or MCA in two mouse strains, male A/J and Swiss-Webster (SWR). MCA predominantly produces K-ras mutations in codons 12/13, whereas urethane affects codon 61 in these mice. Furthermore, in the A/J mice, unlike the findings using NNK/BHT by Matzinger et al. [121], both urethane and MCA induced K-ras mutations in lung tumors, and BHT treatment induced an increased frequency of K-ras mutations in both mouse strains. This result suggests that BHT promotes the activation of K-ras gene in lung tumors in A/J mice.

Other inflammation-inducing agents have also been investigated. Freire et al. [123] studied early molecular changes associated with lung tumorigenesis in a silica-induced chronic inflammatory microenvironment. Female BALB/c mice were treated by oropharyngeal aspiration with a single low dose of the tobacco smoke carcinogen *N*-nitrosodimethylamine (NDMA), silica, a combination of both, or saline [124]. They demonstrated that silica-induced strong inflammatory responses, characterized by increased expression of programmed cell death protein 1 (PD-1), TGF–β1, monocyte chemotactic protein 1 (MCP-1), lymphocyte-activation gene 3 (LAG3), forkhead box P3 (FOXP3), and the presence of regulatory T cells, compared with mice treated with NDMA alone. This created an immunosuppressive microenvironment favoring NMDA-induced development and progression of lung tumors in co-treated mice. There was also an increased incidence of lung tumors and multiplicity in mice treated with NDMA and silica, compared with those treated with NMDA alone. However, the mutational pattern was different between the NDMA-only and NDMA+silica–induced tumors. Specifically, the K-ras mutations in tumors from mice treated with NDMA+silica was primarily G to A transition in codon 12, while A to G transition in codon 61 was the most frequent alteration in mice treated with NDMA alone. Histopathologic analysis showed that tumors from mice treated with NDMA+silica accumulated more anergic and regulatory T cells, characterized by the expression of the PD-1 and Foxp3 markers, respectively, compared with tumors from mice treated with NDMA alone. The predicted reduction in tumoricidal T-cell activity associated with these changes is consistent with the escape of cancer cells from immune elimination. This led the authors to conclude that silica-induced chronic inflammation facilitates the development of preneoplastic lesions and subsequently lung cancer.

## 5.7 Bacteria-Induced Airway Inflammation and Lung Tumorigenesis

COPD is an independent risk factor for lung cancer [125–128], and the airways of COPD patients are commonly colonized by nontypeable *Haemophilus influenzae* (NTHi). Moghaddam et al. [129] showed that repeated exposure of mice to an aerosolized NTHi lysate causes lung inflammation with a profile of mediators and inflammatory cells similar to that observed in patients with COPD. In their follow-up study, they evaluated the effects of this NTHi-induced COPD-like inflammation on mouse models of lung cancer induced by K-ras mutant expression

in airway epithelial cells [130]. NTHi exposure results in leukocyte recruitment and increase in cytokines and chemokines in BAL. Furthermore, this NTHi-mediated, extrinsic COPD-like airway inflammation plays a role in the promotion of lung cancer in one of their mouse models, $CCSP^{Cre}$/LSL–K-$ras^{G12D}$, resulting in a 3.2-fold increase in lung surface tumor number. In addition, NTHi lysate challenge resulted in a shift from macrophage-predominant to neutrophilic airway inflammation in this mouse model, which is associated with significant tumor promotion.

The promoting effect of COPD-like inflammation on lung carcinogenesis was also assessed by using a mouse model with late-onset and low multiplicity lung tumor formation, combining exposure to NNK with NTHi exposure [131]. This mouse model is based on the knockout of the retinoic acid-inducible G protein-coupled receptor [132]. NTHi exposure is associated with activation of NF-κB, release of inflammatory mediators, recruitment of innate (neutrophil and macrophages) and adaptive inflammatory cells, and activation of Hypoxia-inducible factor 1-alpha (HIF-1α), HIF-1α-mediated angiogenesis. Mice exposed sequentially to NNK and NTHi showed a 3.5-fold increase in the multiplicity of surface lesions, compared with mice exposed to NNK alone [131]. Furthermore, a separate study showed that K-ras mutant-mediated lung tumorigenesis and its promotion by COPD-like airway inflammation is associated with significant tumor angiogenesis and activation of HIF-1α [133].

Our group evaluated the effects of inflammation induced by a bacterial component, the lipopolysaccharide (LPS) on lung tumorigenesis caused by exposure of FVB/N mice to NNK [71]. LPS is an endotoxin and a major cell wall component of gram-negative bacteria [134–136]. LPS also is an agonist for innate immune response through activation of the toll-like receptor 4 (TLR4) signaling cascade. Exposure to LPS has been shown to lead to a production of both pro- and anti-inflammatory mediators by myeloid lineage and other cell types including epithelial cells. It has been suggested that LPS is involved in bacterial infection-induced exacerbations of COPD and contributes to the progression of the disease [137]. The recurrent LPS instillation in our mouse model resulted in a promotion of neutrophil and macrophage-dominant chronic inflammation in both LPS + NNK- and LPS-treated mice that is similar to what has been observed in COPD patients.

Inflammatory cell counts in the BAL, including macrophages, neutrophils, and lymphocytes, were significantly increased in the LPS + NNK treatment group. The BAL fluid of chemokines/cytokines, as analyzed by Luminex assays, revealed higher levels of IL-17, CXCL10, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein 1 alpha (MIP-1α), and KC in LPS + NNK than in NNK treatment group [138, 139]. Flow cytometry analysis of the mouse lung tissue revealed that combined LPS and NNK exposure significantly increased $CD4^+$ T cells including Th1, Th17, Tregs, and MDSCs recruitment in the lung. T cell exhaustion related genes, including *Pdcd1, Ctla-4, Tim-3, Lag-3*, and *Foxp3*, and PD-L1 protein were significantly upregulated in the LPS + NNK treatment than NNK treatment. Our data suggest that chronic LPS exposure-promoted and NNK-induced lung tumorigenesis is associated with immunosuppressive tumor microenvironment. The changes include recruitment of Tregs and MDSCs, increased T cell exhaustion, and upregulated PD-1/PD-L1 pathway [138, 139].

We demonstrated that mice treated with LPS alone did not lead to any tumor formation, while mice treated with LPS + NNK developed an averaged 8-fold of synergistically increased incidence of lung tumors, compared with mice treated with NNK alone. There was also an increased rate of K-ras mutation in the tumors of LPS + NNK-treated mice, compared with mice treated with NNK alone (72% vs. 45%, respectively) using FVB/N mice. The mutations all involved the first G/C of the codon 12 of the K-ras gene and consisting of primarily G to A transition. These results suggest that LPS-induced inflammation enhanced the development and progression of K-ras mutant-mediated lung tumorigenesis in LPS + NNK-treated mice [71]. In our lung cancer

model where both LPS and NNK were administered simultaneously, it is likely that LPS-induced inflammation affects the promotion step of NNK-induced lung tumorigenesis. In a later study, Melkamu et al. similarly examined the effects of LPS on NNK-induced lung tumorigenesis in an A/J mouse model. The authors also showed that administration of LPS to NNK-pre-treated mice caused inflammatory responses and a significantly increased tumor multiplicity in the lungs, suggesting that LPS-induced inflammation acts in the promotion stage [139].

## 5.8 Persistent Inflammation Induces KRAS Mutation with Various Genotypes

It has been suggested alveolar macrophages play an important role in mediating the effects of LPS that enters the lungs. During the earliest event in LPS-induced inflammation, LPS is transferred to its cellular receptor complex formed between toll-like-receptor-4, pattern recognition receptor CD14, myeloid differentiation-2, and LPS-binding protein, leading to the signaling of the cellular interior and activation of the alveolar macrophages [140–143]. This leads to a pro-inflammatory cascade defined by the production of specific pro-inflammatory cytokines, such as tumor necrosis factor (TNF), followed by induction of IL-1α and IL-6 [144–146], recruitment of neutrophils to the wound, and a rapid neutrophil infiltration into the lung tissue and airspace [147–149]. In addition to macrophages and neutrophils, other studies suggested that airway epithelial cells, including Club cells and alveolar type II cells [150–153], are capable of producing a variety of pro-inflammatory cytokines that participate in the innate immune responses.

Therefore, extrinsic inflammation induced by various agents promotes lung tumorigenesis initiated by tobacco smoke carcinogens, characterized by a heightened inflammatory response and an increased lung tumor incidence, compared with mice treated with the carcinogen only. However, there were some differences in the K-ras mutational frequencies, patterns, or types in lung tumors without and with treatment with an inflammatory agent (e.g., LPS or BHT) that may indicate that extrinsic inflammation could alter the tumorigenic pathways initiated by a carcinogen. For instance, Matzinger et al. [121] found that the K-ras mutation rate was significantly lower in tumors produced by NNK + BHT than NNK alone. In our study, however, there was a significant increase in both the incidence of lung tumors and the mutation rate of K-ras-positive lung tumors developed in LPS + NNK-treated mice, compared with mice treated NNK only [71, 138, 139]. We also observed a slight change of K-ras mutation type in our study where a subset of G to A transition was identified in mice treated with LPS + NNK but was absent from mice treated with NNK alone [71, 139].

The reasons for the differences in K-ras mutation rates, types, and patterns in lung tumors following different inflammation-promoting agents' treatment are unclear. Mouse strains, carcinogens, inflammatory agents, and their associated inflammatory response patterns could all be a factor. For instance, all K-ras mutations identified in lung tumors from both A/J mice [121] and FVB/N mice [71] treated with NNK only were G to A transition at position 2 of codon 12, but only BHT-treatment altered the tumorigenic pathways from K-ras to a non-K-ras mechanism [121]. Nevertheless, the study by Wang and Witschi suggested that inflammatory agents may not be necessarily a critical factor [122]. Other underlying mechanisms may explain the differences observed. Inflammatory responses, especially induced by agents such as silica and LPS, produce reactive oxygen and nitrogen species that result in oxidative DNA damage, and also inhibition of DNA repair enzymes [154–157]. For instance, reactive oxygen species (ROS) can cause modified bases, apurinic/apyrimidinic sites, and strand breaks. It has been shown that oxygen free radicals and other oxidative agents cause activating K-ras mutations consisting mostly of G to T transversion [158, 159]. A fraction of the K-ras mutations found in tumors from mice treated with LPS + NNK in our study consisted of G to T transversion, compared with none in the NNK-treated group, suggesting that

some of the lung tumors may be initiated by this oxidative pathway following administration to inflammation-promoting agents.

## 5.9 Conclusion

KRAS is a potent oncogene and is mutated in about 25% of all lung cancers. Despite substantial progress made with regard to the cancer treatments, effective cures of the KRAS-associated cancers remain lacking and the KRAS mutation still indicates poor prognosis. Unlike EGFR mutations and ALK rearrangements that now have relatively effective therapies, KRAS mutations are still perceived as "undruggable." Oncogenic KRAS induces inflammation from tumor cells through intrinsic mechanisms, but extrinsic inflammation also results in increased KRAS mutations. A vicious cycle of chronic and persistent inflammation together with increased KRAS mutations creates an immunosuppressive microenvironment that potentiates lung tumorigenesis. Tolerogenic inflammatory cells including T cell exhaustion in KRAS-mutated tumor microenvironment further promote cancer progression. Since KRAS mutation-related lung cancers are strongly associated with inflammation, modulation of inflammatory response could be a target for therapeutic intervention including checkpoint blockade-based immunotherapy.

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