Published in final edited form as:
Toxicol Pathol. 2007 ; 35(1): 75–80.

Overview of the Molecular Carcinogenesis of Mouse Lung Tumor Models of Human Lung Cancer

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Abstract

Lung cancer is the leading cause of cancer death worldwide, and the need to develop better diagnostic techniques and therapies is urgent. Mouse models have been utilized for studying carcinogenesis of human lung cancers, and many of the major genetic alterations detected in human lung cancers have also been identified in mouse lung tumors. The importance of mouse models for understanding human lung carcinogenic processes and in developing early diagnostic techniques, preventive measures and therapies cannot be overstated. In this report, the major known molecular alterations in lung tumorigenesis of mice are reviewed and compared to those in humans.

Keywords

Lung tumor; oncogenes; tumor suppressor genes; K-*ras*; *p53*

Introduction

Human lung cancer can be divided into non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) based on histopathological features. About 80% of human lung cancers are NSCLC, and they are subdivided broadly into adenocarcinoma, squamous cell carcinoma (SCC), and large-cell carcinoma, of which adenocarcinoma is the most prevalent and appears to be increasing in frequency, especially in women and nonsmokers (Husain and Kumar, 2005).

Mouse models have been used for studying human carcinogenesis processes of both NSCLC and SCLC (Meuwissen and Berns, 2005). Most of the earlier models of spontaneous and chemically induced mouse lung tumors more closely resemble human lung adenocarcinoma than other subtypes in morphology and molecular characteristics (Nikitin et al., 2004). Squamous cell and neuroendocrine cell tumor models have also been developed, but they do not develop spontaneously and are discussed later.

Mice of different strains vary markedly in their sensitivity to develop pulmonary tumors. Lung tumor susceptible mice such as strains A and 129 develop a large number of tumors spontaneously with age and produce a strong lung tumor response after treatment with certain carcinogens. B6 (C57BL/6) and C3H mice are relatively resistant to spontaneous development of lung tumors, and BALB/c is considered intermediate in susceptibility (Malkinson, 1985). The B6C3F1 hybrid strain that is commonly used in the National Toxicology Program (NTP) carcinogenesis bioassay has exhibited a strong lung tumor response to certain chemicals, for

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example methylene chloride, chloroprene, and vanadium pentoxide (Kari et al., 1993; National Toxicology Program, 1996, 2001).

The molecular alterations responsible for the chemically induced lung tumor response in B6C3F1 mice have been studied extensively in our laboratories, while other labs have focused on different models. These studies have identified genetic alterations that play a role in lung tumorigenesis and provided clues to mechanisms of carcinogenesis. In addition, epigenetic events that alter the expression of cancer genes have also been studied in these mouse models (Belinsky, 2005).

The carcinogenesis studies that have investigated molecular alterations in mouse lung tumors have provided the basis for new hypothesis driven studies that utilize a gene-targeted approach to induce mouse lung tumors. In recent years transgenic and knockout mice have been created in which lung tumors arise as a result of distinct introduced genetic lesions (Meuwissen and Berns, 2005). Recently, sophisticated site- and stage- specific expression regulated transgenic or knockout mice have been engineered to further understand which genes are specifically responsible for lung tumor formation and progression and how these genes function. Important molecular pathways underlying mechanisms of carcinogenesis in the lung have been revealed and better understood through these targeted transgenic and gene knockout technologies.

Defined mouse models of chemical carcinogenesis have been used to study potential chemoprevention treatments and understand how they work (Yao et al., 2004; Lu et al., 2006). The A/J strain has been utilized primarily for these studies since these mice develop lung tumors rapidly after treatment with certain carcinogens (Mostofi and Larsen, 1951; Dumbell and Rous, 1955; Shimkin and Polissar, 1958). An overview of recent studies is discussed later.

Recently, there is a growing body of gene expression array studies that are adding to our understanding of the molecular mechanisms of human and mouse lung carcinogenesis. Comparisons of gene expression changes in mouse lung tumors and human cancers have revealed some similarities (Bonner et al., 2004). These studies can give strong insights into molecular mechanisms of carcinogenesis. However, they are complicated by the number of different cell populations in the lung that are usually a mixture of cell types in the analysis, while only a small fraction of the cells may be transformed into cancer cells.

K-*RAS* Mutations

For about 2 decades, scientists have been examining tumors for the molecular alterations that are the basis for cancer formation, and *KRAS* was one of the first oncogenes identified. RAS is a critical member of a pathway that transports signals from membrane bound receptor tyrosine kinases to transcription factors that up-regulate growth promotion genes in the cell nucleus. The *RAS* genes consist of a gene family that encode GTPase enzymes, and mutations at critical sites in *RAS* lead to uncontrolled up-regulation of the signaling pathway. Mutations in the *KRAS* proto-oncogene have been identified in about 30% of human lung adenocarcinomas (Reynolds and Anderson, 1991; Reynolds et al., 1992) but are rare in other lung cancer subtypes. Similarly, one of the first molecular alterations to be identified in mouse lung tumors was mutation of K-*ras*, suggesting that mouse lung tumorigenesis may at least partly model human lung cancer. An activating mutation of K-*ras* is a major early event that often occurs in the carcinogenic process of both spontaneous and chemically induced mouse lung tumors, and there is some evidence that it may be a relatively early event in human lung adenocarcinoma formation also (Spivack et al., 1997).

In humans *KRAS* mutations were detected in 25–40% of atypical adenomatous hyperplasia (Cooper et al., 1997), which is a potential precancerous lesion of adenocarcinoma. Thus,

KRAS mutation appears to be an early event in human lung cancer development. K-*ras* mutation occurs in greater than 80% of lung tumors from aging untreated A/J mice and virtually all treated A/J mice, and it has been detected in hyperplasias from these mice (Belinsky et al., 1992; Horio et al., 1996). In contrast, K-*ras* mutations have been identified in only 20% of spontaneous lung tumors from B6C3F1 mice (Sills et al., 1999), and its frequency varies from 20% to near 100% in tumors from treated B6C3F1 mice (Sills et al., 1999) depending on chemical treatment.

Most *KRAS* mutations identified in human lung cancers have been G to T transversions at codon 12, suggesting that benzo(a)pyrene and other carcinogens in tobacco smoke caused specific adduct formation that resulted in the fixation of this mutation pattern in the DNA. Indeed, mouse lung tumors induced by benzo(a)pyrene treatment also exhibited a high proportion of codon 12 G to T mutations in K-*ras* (You et al., 1989; Massey et al., 1995; Sills et al., 1999). It was further revealed that K-*ras* mutation profiles in lung tumors from mice treated with a variety of carcinogens were chemical selective and were often consistent with specific DNA adduct formation by the different chemicals (You et al., 1989; Massey et al., 1995; Sills et al., 1999) or suggested that the tumors might have arisen as a result of indirect DNA damage, oxidative stress, or genomic instability (Sills et al., 1995; Hong et al., 2006).

Interestingly, numerous studies have demonstrated that exposure of pregnant mice to certain chemicals, such as 3′-azido-3′-deoxythymidine (AZT) or 3-methylcholanthrene (3-MC) can increase the incidence of lung tumors in the offspring (Miller et al., 2000; Hong et al., 2006). In the case of the AZT study 25 of 38 (66%) of Swiss (CD-1) mice exposed in utero to AZT had K-*ras* mutations, and they were predominantly G to T transversions in codon 12. This pattern of mutations suggested that exposure to AZT or its metabolites resulted in the formation of specific DNA adducts and mutation fixation in the K-*ras* gene in the offspring.

The importance of K-*ras* mutations in the initiation phase of carcinogenesis has been demonstrated by experiments that created transgenic mice carrying a mutant K-*ras* allele (Johnson et al., 2001). These mice developed a range of tumor types, especially early onset lung tumors, suggesting that K-*ras* mutation can initiate mouse lung carcinogenesis. Another transgenic mouse lung cancer model that expressed mutant K-*ras* under the control of doxycycline specifically in pulmonary alveolar type II cells revealed that continued production of the mutant K-*ras* was required to maintain the malignant phenotype even in animals with deficient *p*53 or *p16* tumor suppressors (Fisher et al., 2001). K-*ras* mutations have been identified in early lesions, but metastatic lesions have not been detected commonly in these studies, suggesting that alterations in other cancer genes are required for progression and metastasis.

While K-*ras* appears to play a critical role in mouse and human lung adenocarcinoma formation, its function is complex and its role is only beginning to come to light. In 2001, Zhang et al. (2001) proposed that wild-type K-*ras* may be a mouse lung tumor suppressor gene. In that study chemical-treated mice carrying one disrupted allele of K-*ras* developed a high incidence of lung tumors, and the remaining K-*ras* allele in the lung tumors was mutated. One of the clues that led to these experiments was the finding of loss of heterozygosity (LOH) on chromosome 6 in the region of K-*ras* in lung tumors from B6C3F1 mice with K-*ras* mutations (Hegi et al., 1994; Zhang et al., 2001). In addition, K-*ras* map kinase activation correlated strongly with K-*ras* mutation and LOH in a set of lung tumors from B6C3F1 mice (Devereux et al., 2002).

K-*ras* is known to map closely to the mouse *Pas1* (pulmonary adenoma susceptibility) gene, and genetic polymorphisms in the 2nd intron between inbred mouse strains correlate with susceptibility (You et al., 1992). However, this small region of mouse chromosome 6 is very

complex, and other candidates for *Pas1* have also been identified (Wang et al., 2005). At this time, the role of K-*ras* in mouse lung tumor susceptibility and tumor formation has not been entirely deciphered.

p53 Mutation

Inactivation of the *p53* gene plays a fundamentally important role in the pathogenesis of lung cancer. The *p53* tumor suppressor functions in a network of pathways, playing critical roles in cell cycle checkpoints, apoptosis, DNA repair and recombination (Sengupta and Harris, 2005). It is induced following cellular stress to maintain genomic stability and is lost or rendered dysfunctional during carcinogenesis in many systems. Mutation of *p53* has been frequently reported in both SCLC (75%) and NSCLC (50%) (Takahashi et al., 1989), but adenocarcinomas exhibit *p53* mutations less frequently than SCC or other histological types of lung tumors (Calvez et al., 2005).

Association between smoking and mutation patterns of *p53* has been reported for lung cancers (Calvez et al., 2005), suggesting that there may be different mechanisms or pathways depending on exposure to tobacco. In spontaneous and chemically induced mouse lung tumors, *p53* is not commonly mutated (Hegi et al., 1993), and when it is mutated, it is a late event (Horio et al., 1996). Hegi et al. (1993) identified *p53* mutations in only 4/54 methylene chloride induced lung carcinomas examined from B6C3F1 mice, and those tumors also exhibited LOH at the *p53* locus.

Overexpression of *p*53 by immunochemical staining was heterogeneous and was found in those 4 tumors and in a focal area of 1 other tumor. In another study *p53* overexpression in aflatoxin B1 induced AC3F1 mouse lung tumors was found to be highly heterogeneous (39). Laser capture microdissection of immunohistochemically positive cells was utilized to identify *p53* mutations. Twenty-six mutations were identified in the microdissected regions, and concordance between staining and mutation was 72%. In addition, the *p53* mutations detected did not occur specifically at G:C basepairs as was found for all K-*ras* mutations in these tumors (Donnelly et al., 1996).

These studies provide evidence that *p53* mutations are late events in mouse lung carcinogenesis and most likely result from indirect DNA damage or genomic instability. In a recent study utilizing *p53* mutant mouse models, *p53* was found to play an important role in progression, especially in K-*ras* induced lung carcinogenesis (Jackson et al., 2005).

Other Cancer Genes May Influence Development of Mouse Lung Tumors

The p16-Ink4a CDK4 inhibitor encoded by *CDNK2A* plays a critical role in the G1/S cell cycle checkpoint by controlling the CDK4-cyclin D1 complex that regulates RB expression. It is thought that most cancers have a disruption in the RB pathway that results in acceleration of cell cycle progression. Loss of RB protein occurs in 15–30% NSCLC with a higher frequency in late-stage NSCLC than in early-stage tumors, suggesting a possible association with tumor progression (Xu et al., 1996). However, loss of *p16* function appears to be a more common occurrence in tumors, and it is an early event in lung cancer (Belinsky et al., 1998).

Allele loss, mutation and methylation of p16 have been detected in many human cancers. However, while mutations of *p16* are common in melanomas, in human lung cancers promoter methylation and allele loss predominate in those tumors where p16 loss has been identified. That also appears to be the case for mouse lung tumors (Patel et al., 2000; Tam et al., 2003). In a study of spontaneous and methylene chloride-induced lung adenocarcinomas from B6C3F1 mice, *Cdkn2a* promoter region methylation was detected in DNAs from 12 of 17 tumors, but not from normal lung (Patel et al., 2000). In a similar study on lung tumors from

AC3F1 mice treated with aflatoxin B1, LOH in the region of the *Cdkn2a/p19Arf* gene loci on chromosome 4 occurred in 22/74 (30%) of the tumors.

In addition, 51/61 (83%) of the tumors showed at least partial methylation of CpG sites in the *Cdkn2a* promoter and 43 of 49 (88%) exhibited at least partial methylation of the *p19Arf* promoter (Tam et al., 2003). Interestingly, disruption of *p16Ink4a*, *p19Arf*, and *p53* did not show strong correlations, suggesting inactivation of these genes is independent and that they may function in independent as well as cooperative pathways. In other studies the incidence of spontaneous lung tumors was not increased in *p53*, *Rb* or *p16 Ink4a* mutant mice, although combinations of these genetically targeted mice with mutant K-*ras* did show increased incidences of lung tumors (Fisher et al., 2001; Wang et al., 2006).

The death associated protein (DAP)-kinase appears to play a role in apoptosis by activating p53 in a p19 ARF-dependent manner to inhibit cell transformation (Raveh et al., 2001). Loss of expression of DAP-kinase by promoter methylation may play a role in early (Tang et al., 2000) and late stages of (Kim et al., 2001) human NSCLC and in early steps in mouse lung tumorigenesis (Pulling et al., 2004). Methylation was observed in 40–60% of mouse lung tumors induced by cigarette smoke, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), vinyl carbamate, or methylene chloride as well as in hyperplasias associated with NNK exposure. The frequency of methylation in the mouse lung tumors was comparable to that reported for smoking-associated human lung cancer (Belinsky, 2005).

Enhanced expression of cyclooxygenase 2 (COX2) has been observed in human lung adenocarcinomas (Hida et al., 1998), and epidemiological studies have shown that NSAIDS such as aspirin that inhibit COX activity significantly reduce the risk of lung cancer (Schreinemachers and Everson, 1994). Two studies have followed the expression of Cox2 (and/or Cox1) during lung carcinogenesis in A/J mice (Bauer et al., 2000; Wardlaw et al., 2000). Immunostaining was detected in normal alveolar and bronchial cells and in some but not all adenomas and carcinomas in lung tumor susceptible mice.

Another study demonstrated that NSAID inhibitors of Cox2 inhibited mouse lung tumor formation and involved both induction of apoptosis and inhibition of Cox2 expression (Yao et al., 2000). Overexpression of Cox2 does not seem to be obligatory for progression to malignancy in this model, although it may be important in early stages of mouse lung tumor formation as well as play a prognostic role at early stages of human lung cancer (Maxcaus et al., 2006).

Up-regulation of telomerase occurs frequently in both NSCLC (80%) and in SCLC (100%) and has been detected in precancerous lung tissue (Osada and Takahashi, 2002). Telomerase activation also occurs during mouse lung carcinogenesis (Ohno et al., 2001). Increased telomerase activity was detected during early and late urethane-induced tumorigenesis in A/J mice and was independent of *p53* gene alterations.

The allelic loss of chromosome 3p is one of the most frequent genetic alterations in both SCLC (90%) and NSCLC (70%). It is detectable even in histologically normal or mildly abnormal lung epithelium in lung cancer patients and healthy former or current smokers. The region of 3p21.3 harbors a number of candidate tumor suppressor genes including a RAS-related gene, *RASSF1A*, the loss of which may be important in early stages of human lung carcinogenesis (Li et al., 2003). Methylation of the *RASSF1A* promoter was detected in 55% of lung adenocarcinomas, 25% of large cell carcinomas, and 25% of squamous cell carcinomas (Li et al., 2003). That study also found that the majority of tumors with *KRAS* mutations lacked RASSF1A inactivation. To date there are no reports of *Rassf1a* mutation or promoter methylation in lung tumors in mice. However, *Rassf1a* knockout mice were susceptible to spontaneous tumor formation in old age, and when treated with the lung carcinogen urethane,

exhibited and increased tumor multiplicity and tumor size relative to control mice (Tommasi et al., 2005). These data support a role for Rassf1a in lung tumor suppression.

Besides the studies on individual major cancer genes that have uncovered important clues to molecular mechanisms of lung cancer, advances in global gene expression analysis and bioinformatics have enabled scientists to examine changes in expression of thousands of genes and many pathways in single experiments. Many studies on lung cancers have been able to dissect patterns of gene expression that were specific to tumor subtypes, smoking status, and prognosis (Miura et al., 2002). There have been a number of recent studies that have compared global gene expression changes in mouse lung tumors and human lung cancers.

For example, one study found a similarity of gene-expression patterns of many cancer-associated genes between mouse lung tumors and human lung adenocarcinomas (Bonner et al., 2004). In another study a gene expression signature of K-*ras* activation in a mouse model of lung tumors uncovered a *KRAS* gene expression profile in human lung cancer that was not revealed when analyzing the human tumors alone (Sweet-Cordero et al., 2005). Thus, mouse models are providing valuable information that will help in understanding human lung cancer.

Squamous and Small Cell Models of Lung Cancer

Each type of tumor appears to have not only preferential etiology but also proceed from different mechanisms of carcinogenesis associated with distinct patterns of genetic lesions. Until recently few models of lung cancer other than adenocarcinoma existed. Models of squamous cell lung carcinoma have been developed after intubation with methyl carbamate (Nettesheim and Hammons, 1971) or skin painting of mice with N-nitroso-tris-chloroethylureas (Rehm et al., 1991). Skin-painting with these compounds induced squamous cell carcinomas of the lung in susceptible strains of mice including SWR/J, NIH Swiss, BALB/c, A/J and FVB/J but not in resistant strains (Wang et al., 2004). Meuwissen et al. (2003) have induced a SCLC model by conditional knockout of *p53* and *Rb*. Recently, several other mouse models of human lung cancer have been developed, especially by transgenic technologies and these models are reviewed in detail elsewhere (Meuwissen and Berns, 2005).

Identification of Human Carinogens and Chemoprevention Models

Mouse models have been utilized by the National Toxicology Program and other groups to identify human carinogens and understand risks of exposure. Many chemicals in the Report on Carcinogens (NTP) are listed as known human carcinogens based in part on supporting evidence from NTP carcinogenicity tests in mouse models. One criticism of the animal tests has been the inability to demonstrate carcinogenicity of tobacco smoke in mice. However, a new study has shown that lifetime exposure of high doses of cigarette smoke strongly increases the lung tumor incidence in B6C3F1 mice (Hutt et al., 2005).

Mouse lung tumor models have also been utilized to test chemopreventive strategies (You and Bergman, 1998; Chung, 2001), and recently gene expression profile studies have been conducted to understand the observed effects. For example, budesonide, a glucocorticoid, inhibited tumor multiplicity by 70% and total tumor load by 94% in A/J mice treated with benzo(a)pyrene (Yao et al., 2004). Gene expression analysis indicated that budesonide modulated growth arrest, apoptosis, and interference pathways that likely resulted in its chemopreventive effect. In another study green tea was found to be a strong chemopreventive agent against lung tumors in A/J mice (Xu et al., 1991). Gene expression studies have suggested that major pathways involved in cell signaling, cell proliferation, and transcription in the lungs and lung tumors are affected by the green tea consumption (Lu et al., 2006).

Conclusions

Lung cancer is currently the most frequently diagnosed major cancer in the world and the most common cause of the cancer mortality worldwide. The high mortality is largely due to the late stage of diagnosis and the poor response to therapy. Mouse lung tumors are not identical to human lung cancers, but they share many genes and pathways for lung cancer development. Since human lung cancers have a wide range of gene-expression patterns depending on the type, it is important to find proper mouse models for each type of human lung cancer. The use of mouse models is adding to our understanding of lung cancer biology by uncovering the critical molecular pathways responsible for each stage of tumor formation and progression. A better understanding of the carcinogenesis process will lay a foundation for future development of prevention and therapy for lung cancer as well as tools providing sensitive diagnosis and reliable prognosis.

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