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# Genetic Alterations in Mouse Lung Tumors: Implications for Cancer Chemoprevention

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**Abstract** Specific genetic alterations affecting known tumor suppressor genes and proto-oncogenes occur during mouse lung tumorigenesis. These include mutational activation of the K-*ras* gene, commonly seen at a frequency of about 80% in both spontaneously occurring and chemically induced adenomas and adenocarcinomas of the lung, suggesting that it is an early event that persists into malignancy. Allelic loss of the p16 tumor suppressor gene also is a frequent event, occurring in about 50% of mouse lung adenocarcinomas, but rarely in lung adenomas, suggesting that it may play a role in malignant conversion or progression of lung tumors. Other genetic alterations detected in mouse lung tumors include reduced expression of Rb and p16, and increased c-*myc* expression. Alterations of these genes are also common in the genesis of human lung cancer. Genetic linkage analysis to identify human lung cancer susceptibility genes is difficult due to the genetic heterogeneity and exposure to environmental risk factors. The mouse lung tumor model has become a valuable alternative for identifying such genes. Recently, loci responsible for mouse lung tumor susceptibility have been mapped to chromosomes 6, 9, 17, and 19, while those linked to lung tumor resistance have been mapped to chromosomes 4, 11, 12, and 18. Known candidate susceptibility or resistance genes include the K-*ras* proto-oncogene on chromosome 6, and the p16 tumor supressor gene on chromosome 4. With evidence of considerable overlap between the genetic alterations that underlie human and mouse lung tumorigenesis, the mouse lung tumor model has been expanded to include pre-clinical screening of chemopreventive agents against human lung cancer. Studies on the modulation of genetic defects in mouse lung tumors by known and potential chemopreventive agents should further the goal of developing an effective prevention and treatment of lung cancer. J. Cell. Biochem. Suppls. 28/29:49–63. 

**Key words:** mouse lung; genetic alteration; aberrant gene expression; surrogate endpoint biomarkers; cancer chemoprevention

Lung cancer is the leading cause of cancer mortality in both males and females in developed countries [1]. Epidemiological studies have indicated that approximately 85% of all lung cancer deaths in the United States are associated with tobacco smoking [2]. Relative risk for lung cancer is increased in smokers at least 13-fold and in passive smokers by 1.5-fold, with a linear relationship between the number of cigarettes smoked and lung cancer risk [3,4]. Approximately 50 of the chemicals in cigarette smoke, including polyaromatic hydrocarbons, nitrosamines, and aromatic amines, have been shown to be mutagenic or carcinogenic [5]. Additional environmental and occupational risk factors for lung cancer include exposure to asbestos, arsenic, chromium, nickel, and radon [6]. The mouse lung tumor bioassay was developed more than 50 years ago to identify potential lung carcinogens, and has been instrumental in demonstrating the carcinogenicity of a wide range of chemicals [7].

Lung cancer, like other types of cancer, develops as a multistage process involving the accumulation of genetic alterations that affect key proto-oncogenes and tumor suppressor genes. Many of the known changes are common to both human and mouse lung tumors. Figure 1 shows the genetic alterations detected during mouse lung tumorigenesis. To date, the most common alterations in mouse lung tumors have affected genes associated with numerous cancer types. Mutation of the K-*ras* proto-oncogene

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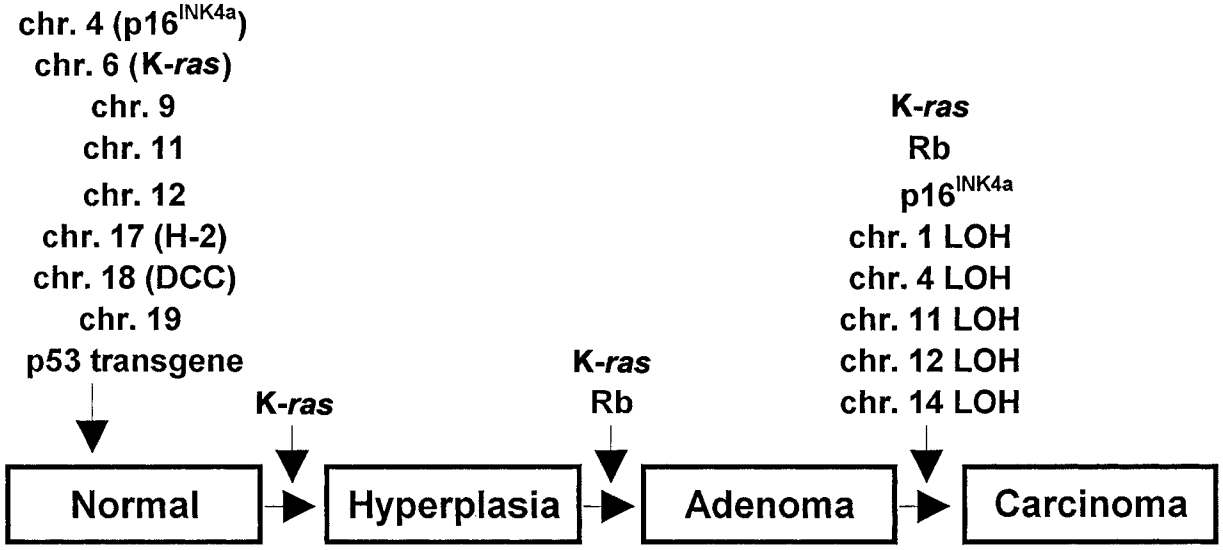


**Fig. 1.** Genetic alterations found during mouse lung tumorigenesis.

is seen in about 80% of mouse lung adenomas and adenocarcinomas and is the principal candidate for a major susceptibility gene on chromosome 6 [8,9]. Other frequent alterations affect tumor suppressor genes p16 and p53, which have been associated with specific stages of both human and mouse lung tumorigenesis [10,11]. Aberrant expression of c-*myc*, Rb and p16 gene also have been found in mouse lung tumorigenesis [12] (Liu et al., unpublished data). Finally, newly developed screening methods have revealed numerous genetic changes, suggesting that additional unidentified genes may also contribute to mouse lung tumorigenesis (Lin et al., unpublished data).

Over the past decade, a number of genes predisposing to the development of specific types of cancer have been identified [13]. Similarly, there is evidence that the susceptibility of the human population to different forms of lung cancer follows a pattern of autosomal dominant Mendelian inheritance [14–18]. However, the pervasiveness of lung carcinogens in our environment has made it difficult to accurately identify familial clusters of lung cancer patients necessary to identify predisposing genes. The use of mouse models imparts control over environmental factors that confound human studies on the genetics of lung cancer. Inbred mouse strains are variable in genotype, as well as in susceptibility to lung cancer, ranging from the very susceptible A/J strain to the very resistant C57BL/6J strain [19]. Genetic studies have taken advantage of these strain differences to map lung tumor susceptibility and resistance genes to specific chromosome locations [20]. Thus far, linkage has been demonstrated for loci on chromosomes 4, 6, 9, 11, 12, 17, 18, and 19 [9, 21–29]. Knowing which genes predispose or underlie the development of mouse lung cancer is of considerable interest to further understanding of human disease. A number of candidate susceptibility and resistance loci now exist based on their chromosomal location relative to the regions of strong linkage, and in some cases, also based on prior demonstrated involvement in mouse lung tumorigenesis. Chemopreventive strategies may use molecular changes that control the genesis of lung cancer as targets or intermediate endpoint biomarkers. Progress toward understanding these changes is reviewed below.

## SUSCEPTIBILITY OF MOUSE STRAINS TO CHEMICAL LUNG TUMOR INDUCTION

Although the majority of lung cancer cases are associated with cigarette smoking and environmental exposure, increasing evidence suggests that individuals differ in their susceptibility to environmental factors. An increased familial risk for lung cancer has been observed within lung cancer probands [14]. Further segregation analyses provided evidence that susceptibility of the human population to different forms of lung cancer follows a pattern of autosomal dominant Mendelian inheritance [14–18]. However, there have been no reports on localiza-

tion and identification of human lung cancer susceptibility gene(s).

Genetic differences between mouse strains are analogous to genetic differences within the human population, making the mouse lung system an excellent tool for studying genetic components underlying tumor development and susceptibility [30]. Inbred mouse strains show widely different susceptibilities to both spontaneously occurring and chemically induced lung tumor formation [31]. This susceptibility is intrinsic to the lung itself, as shown by classic experiments involving lung explants from sensitive and resistant mice, which showed that tumors developed after carcinogen treatment only in lungs of the sensitive mouse strain [32, 33]. Matings of sensitive A/J and resistant C57BL/6J mice produce $F_1$ and $F_2$ offspring which have intermediate sensitivity to tumor induction, indicating that this phenotype is conferred by more than one gene [34]. The production of recombinant inbred (RI) lines of A/J and C57BL/6J mice and subsequent analysis of their sensitivities to tumorigenesis originally suggested that three genes, one major and two minor, were involved in determining the sensitivity to mouse lung tumor development [34]. Subsequent linkage studies were conducted to identify pulmonary adenoma susceptibility (*Pas*) and pulmonary adenoma resistance (*Par*) loci, with tumor multiplicity and size used as quantitative traits. These results have revealed the polygenic nature of the predisposition to tumor induction of the mouse lung. Listed in Table I and outlined below are the quantitative trait loci (QTL) that have been mapped to lung tumor susceptibility/resistance in various mouse crosses.

### Pas1

A major susceptibility locus was mapped to distal chromosome 6 in (A/J x C3H/HeJ) $F_2$ mice, and was termed the *Pas1* locus. This locus produced a maximum logarithm of the likelihood ratio (LOD) score of 9 and accounted for approximately 45% of the observed phenotypic variance [9]. A LOD score of 3 or greater is considered significant for linkage. Corroborating results were obtained in comprehensive linkage studies using (A/J x C57BL/6J) $F_2$ (60% of variance), (A/J x C57BL/6J) x C57BL/6J (16% of variance), (A/J x *M. spretus*) x C57BL/6J (34% of variance), and AxB and BxA RI mice (51% of variance) [21–23, 35]. The QTL for *Pas1* showed tightest linkage at the locus of the K-*ras* gene, which became the principal candidate gene for *Pas1* based on the understanding that K-*ras* gene activation is an early event often found in

**TABLE I. Mouse Lung Tumor Quantitative Trait Loci (QTL)**

| QTL | Chr | Cross[a] | Variance (%) | Candidates | References |
|---|---|---|---|---|---|
| *Pas 1* | 6 | A × B & B × A RI | 51 | K-*ras* | [23] |
| | | (A/J × C3H/He)$F_2$ | 40 | K-*ras* | [9] |
| | | (A/J × B6)$F_2$ | 60 | K-*ras* | [21] |
| | | (A/J × B6) × B6 | 16 | K-*ras* | [22] |
| | | (A/J × *M. spretus*) × B6 | 40 | K-*ras* | [35] |
| *Pas 2* | 17 | (A/J × B6)$F_2$ | 7 | TNF α/β | [21] |
| | | A × B & B × A RI | 29 | TNF α/β | [23] |
| *Pas 3* | 19 | (A/J × B6) × B6 | 3 | | [22] |
| | | (A/J × B6)$F_2$ | 2 | | [21] |
| | | A × B & B × A RI | 26 | | [23] |
| *Pas 4* | 9 | (A/J × B6)$F_2$ | 4 | | [21] |
| *Pas 5* | 10 | A × B & B × A RI | 22 | | [23] |
| *Par 1* | 11 | (A/J × *M. spretus*) × B6 | 15 | | [26] |
| *Par 2* | 18 | (A/J × BALB/c) × A/J | 38 | DCC | [27] |
| | | (A × BALB/c)$F_2$ | ~50 | DCC | [25] |
| *Par 3* | 4 | (A × BALB/c)$F_2$ | ~10 | $p16^{INK4a}$ | [24] |
| *Par 4* | 12 | SM × A RI | | | [29] |
| *Sluc 1* | 19(D19MIT9) | (OcB-9 × 020)$F_2$ | | | [28] |
| *Sluc 2* | 2(D2MIT56) | (OcB-9 × 020)$F_2$ | | | [28] |
| *Sluc 3* | 6(D6MIT218) | (OcB-9 × 020)$F_2$ | | TNF R1 | [28] |
| *Sluc 4* | 11(D11MIT15) | (OcB-9 × 020)$F_2$ | | p53 | [28] |

[a]B6 = C57BL/6; B = C57BL/6; A = A/J.

both spontaneously occurring and chemically induced mouse lung tumors, and that polymorphisms detected in K-*ras* promoter and enhancer regions in different mouse strains correlate with their susceptibility to chemical induction of lung tumors [8,36]. Also, these polymorphisms seem to be responsible for the observed allele-specific expression of the K-*ras* allele in hybrid mice, which leads to allele-specific activation of the K-*ras* gene [19,37]. Finally, genetic linkage analyses indicate a major locus at this location only when parental mice have distinct K-*ras* genotypes; for example, studies on (A/J x BALB/cByJ) x A/J and (A/JO1aHsd x BALB/cO1aHsd) $F_2$ mice showed no linkage between lung tumor formation and *Pas1* [25,27], indicating that BALB/c and A/J are genetically alike at the *Pas1* locus. Both of these strains possess the same K-*ras* variant.

### *Pas2*, *Pas3*, and *Pas4*

Loci shown to positively modulate the effect of *Pas1* were mapped to chromosomes 9, 17, and 19 [21,22]. Linkage to the site of the putative *Pas2* locus on chromosome 17 was observed in (A/J x C57BL/6J) $F_2$. This locus accounted for 7% of the total variance in phenotype. The location of the *Pas2* locus is homologous to human chromosome 6p21; possible candidates at this location are the genes for tumor necrosis factor (TNF) $\alpha$ and $\beta$. Similarly, linkages to lung tumor susceptibility were also seen at markers on chromosome 19 (*Pas3*), accounting for 3% of the phenotypic variation in a study on (A/J x C57BL/6J) x C57BL/6J mice, and 2% of the explained phenotypic variation when (A/J x C57BL/6J) $F_2$ mice were used. In this latter study, suggestive linkage to a locus on chromosome 9 (*Pas4*) was determined to explain 4% of the total phenotypic variance [21].

### Par1

A lung tumor resistance locus, *Par1*, was recently mapped in (A/J x *M. spretus)* x C57BL/6J mice to chromosome 11 overlapping the retinoic acid receptor-$\alpha$ (*Rara)* gene locus [26]. Contributed by the *M. spretus* allele, *Par1* gave a maximum LOD score of 5.3 and accounted for 23% of phenotypic variance when co-expressed with the highly penetrant *Pas1* allele of the A/J strain. In mice carrying the *M. spretus* instead of the A/J allele of the *Pas1* gene, the resistant effect of *Par1* on tumor incidence, multiplicity and volume was lessened by about one-half. Thus, *Par1* behaves like a modulator of *Pas1*, to some degree subduing the dominant effect of *Pas1* on lung tumorigenesis [26].

### Par2

Linkage studies in (A/J x BALB/cByJ) x A/J and (A/JO1aHsd x BALB/cO1aHsd) $F_2$ mice revealed significant linkage on chromosome 18 at microsatellite marker D18MIT103. A LOD score of 12.2 was reported at this locus, with a phenotypic variance of 38% for resistance to tumor induction [27]. This locus was termed *Par2*. In our analysis of (A/JO1aHsd x BALB/cO1aHsd) $F_2$ mice, *Par2* had a significant linkage to lung tumor resistance and produced a maximum LOD score of 11 [25]. The greatest linkage occurred at the site of the *DCC* tumor suppressor gene [25,27]. The *DCC* gene was identified on human chromosome 18q21 as a target of somatic mutation and allelic loss in colorectal carcinomas [38]. Since its identification, many studies have shown that its loss is common to several other types of cancer including those of breast, prostate, esophagus, endometrium, pancreas, stomach, and brain [39,40]. However, the role of *DCC* as a tumor suppressor is still in question since *DCC* deficient mice did not develop any tumors. This gene codes for a transmembrane protein comprised of four immunoglobulin-like and six fibronectin Type III-like domains. Recently, *DCC* was shown to function in the nervous system as a neurin receptor or receptor component that mediates neurin-directed axon outgrowth [41]. Of potential significance to lung cancer, *DCC* has been shown to suppress the malignant phenotype of transformed human epithelial cells [42]. The human and mouse *DCC* proteins share 96% identity, and their genes are tightly linked to two other candidate tumor suppressor genes, with human homologues named Deleted in Pancreatic Cancer 4 (DPC4) and MADR2/JV18–1. Recently, we observed no sequence polymorphisms in the *DCC* gene between the A/J allele and BALB/cJ allele, or difference in allele-specific expression of the *DCC* gene, that suggests against its inclusion as a candidate lung tumor resistant gene (Lin et al., unpublished data). Similar observations have also been made for DPC4 and MADR2/JV18–1 [25].

### Par3

We also observed linkages to susceptibility on chromosome 4 (D4MIT77) (LOD score = 3.0) using (A/JO1aHsd x BALB/cO1aHsd) $F_2$ mice [24]. *Par3* seems to have a stronger resistance to lung tumor induction when co-expressed with the A/J allele of *Par2* [24, 43]. Linkage on chromosome 4 was strongest at a marker recombinationally inseparable from the p16 tumor suppressor gene locus; the BALB/cJ allele at this locus is associated with sensitivity to lung tumor formation. p16 has been shown to specifically inhibit serine/threonine protein kinase activity of cyclin D-dependent kineses CDK4 and CDK6 [44]. The principal target of these kinases is the retinoblastoma protein (pRb). When phosphorylated by CDK4 and/or CDK6, pRb is inactivated and rendered incapable of maintaining its inhibitory sequestration of the E2F family of transcription factors. Upon release, these transcription factors activate the transcription of genes important in cell growth, such as dihydrofolate reductase, thymidine kinase, and cyclin A and E [45]. Overexpression of members of the E2F family have been shown to override p16-induced cell cycle arrest [45]. p16 and pRb are both negative regulators of the passage of cells through the G1 phase of the cell cycle; inactivation of either is common to several types of cancer [46].

There is evidence to suggest that the p16 gene is a candidate for *Par3*. Two variants of p16 that differ at amino acids 18 and 51 were shown to exist in mice [43]. The A/J and BALB/c strains represent the two groups of variant strains. These observations suggest that the BALB/c variant of p16 may confer resistance to lung tumorigenesis.

### Par4

A locus conferring resistance to urethane-induced lung tumorigenesis was mapped to chromosome 12 (LOD score 6.4) using SM x A RI strains of mice [29]. One potential candidate gene for *Par4* is protein kinase Cη which is expressed only in skin and lung tissues.

### Sluc1, Sluc2, Sluc3, and Sluc4

Complex interactions between QTLs on chromosomes 19 (distinct from *Pas3*) and chromosome 2, and between loci on chromosome 6 (distinct from *Pas1*) and chromosome 11 (distinct from *Par1*), were suggested to influence lung tumor size or rate of growth in (OcB-9 x 020) $F_2$ mice [28]. These loci were termed Susceptibility to lung cancer 1 (*Sluc1*), *Sluc2, Sluc3,* and *Sluc4*, respectively. OcB-9 is a recombinant congenic strain that shares 87.5% of its genetic identity with the O20 inbred strain, and 12.5% with strain B10.O20. This study used multiple-QTL models (MQM) mapping allowing for interactions between QTLs. It was observed that within an interaction, the affect on tumor size depended on the genotype of each locus. For example, the affect of *Sluc1* on tumor size was large when *Sluc2* was homozygous for O20, but small when *Sluc2* was homozygous for B10.O20. The effect of *Sluc3* on tumor size was large when *Sluc4* was homozygous for the O20 allele and small when *Sluc4* was homozygous for the B10.O20 allele. Also, susceptibility to large tumors was seen when *Sluc1* and *Sluc2* were both homozygous for O20, but significant resistance was seen when *Sluc1* was homozygous BI0.O20 and *Sluc2* was homozygous O20. Similarly, susceptibility to large tumors was significant when *Sluc3* was homozygous O20 and *Sluc4* was homozygous B10.O20. Significant linkage with very small tumors occurred when both *Sluc3* and *Sluc4* were homozygous for B10.O20. Interestingly, the interacting loci *Sluc3* and *Sluc4* map to the same approximate locations as the genes for TNF receptor 1 and the p53 tumor suppressor, respectively, whose functions could conceivably interact to affect tumor volume.

## ONCOGENES

K-*ras* mutations detected during mouse lung tumorigenesis tend to occur early and persist into malignancy [8]. The mutation spectra in the K-*ras* gene of these tumors induced by different carcinogens are clearly distinct and consistent with the expected mutagenic specificity of the carcinogens [8]. As shown in Table II, we observed an almost exclusive occurrence of activated mutations in the A/J K-*ras* allele in lung tumors of F1 hybrid offspring produced from crosses between strains of mice that are susceptible and resistant to lung tumor formation [36]. DNA sequence variations between inbred mouse strains have been detected in the second intron [19], 5'-promoter region (Zhang et al., unpublished data) and 3'-untranslated region (UTR) [47] of the K-*ras* gene, which may contribute to the constitutively higher expression of the A/J allele relative to the C3H allele.

**TABLE II. Allele-Specific Expression and Localization of the K-*ras* Oncogene Detected in hybrid Mouse Lung Tumors***

| Treatment | $F_1$ hybrids | Activated K-*ras* gene | Allele-specific expression | | Allele-specific location | | References |
|---|---|---|---|---|---|---|---|
| | | | Alleles | ↑ (fold) | Others | A/J or BALB/c | |
| None | (C3H/HeJ × A/J)$F_1$ | 20 | A/J > C3H | ND | 2 | 18 | [19, 85] |
| NNK | (C3H/HeJ × A/J)$F_1$ | 7 | A/J > C3H | 2–50 | 0 | 7 | [19, 85] |
| VC | (C3H/HeJ × A/J)$F_1$ | 13 | A/J > C3H | 2–50 | 0 | 13 | [19, 85] |
| NNK | (A/J × C3H/HeJ)$F_1$ | 19 | A/J > C3H | 2–50 | 0 | 19 | [19, 85] |
| VC | (A/J × C3H/HeJ)$F_1$ | 15 | A/J > C3H | 2–50 | 0 | 14 | [19, 81] |
| DMN | (C3H/HeJ × A/J)$F_1$ | 15 | A/J > C3H | 2–50 | 0 | 14 | [37] |
| B(*a*)P | (C3H/HeJ × A/J)$F_1$ | 15 | A/J > C3H | 2–50 | 0 | 15 | [37] |
| NNK | (A/J × TSGp53)$F_1$ | 38 | A/J > TSGp53 | 10–20 | 0 | 38 | [47] |
| MNU | (A/J × *M. Spretus*)$F_1$ | 12 | A/J > *M. Spretus* | 1.7–12 | 0 | 12 | [51] |
| VC | (C57BL/6J × BALB/cJ)$F_1$ | 18 | N.D. | N.D. | 3 | 15 | [49] |
| $AFB_1$ | (A/J × C3H/HeJ)$F_1$ | 76 | N.D. | N.D. | 3 | 73 | [50] |
| ENU | RCS | 134 | N.D. | N.D. | 1/71 | 13/63 | [86] |

*N.D., not determined. NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; VC, vinyl chloride; DMN, dimethylnitrosamine; B(*a*)P, benzo(*a*)pyrene; MNU, *N*-methylnitrosourea; $AFB_1$, aflatoxin $B_1$; ENU, *N*-ethylnitrosourea; RCS, recombinant congenic strains.

In fact, differential protein-binding patterns were observed in gel mobility-shift experiments between the duplicated 37 bp sequence of the $K^R$ allele (K-*ras* allele identical to that of the resistant C3H/HeJ strain) and the single 37 bp sequence of the $K^S$ and $K^I$ alleles (K-*ras* alleles identical to that of the susceptible A/J strain) [19]. DNase I footprinting assays revealed protein binding sites in the second intron of the K-*ras* gene, which corresponded to the tandem repeat sequences. In a separate study, another protein-binding site located downstream (nucleotides 463–509) from the tandem repeat region was identified and shown by DNase I footprinting to be a protein binding site in the second intron [48]. Southwestern blot analysis indicated that these two repetitive regions could be involved in binding with the same regulatory complex. Furthermore, gel mobility-shift studies showed differential protein-binding patterns between the $K^R$ allele and the $K^S/K^I$ allele. These results suggest that the repetitive sequences in the second intron could play a role in differential transcriptional regulation of the K-*ras* gene.

The $K^S$ allele was also expressed at significantly higher levels than $K^R$ alleles in lung tissue from hybrid mice (Table II) [19,37,47]. For example, $K^S$ was 1.4 to 2 times higher than $K^R$ in lung tissues from 4–8-week-old untreated (C3H/HeJ X A/J) $F_1$ or (A/J X C3H/HeJ) $F_1$ mice, and 2 to 12 times higher in (A/J X C57BL/6J) $F_1$ hybrid mouse lung tissues harvested over a 20-week period. Furthermore, oncogenic K-*ras* from the A/J parent is expressed 2–50 times over the normal level of C3H allele in lung tumors (Table II). As alluded to above, lung tumors from (C3H/HeJ X A/J) $F_1$ or (A/J X C3H/HeJ) $F_1$ mice nearly always display activating mutations of the $K^S$ allele. Similar results have been observed in lung tumors from (A/J X TSGp53) $F_1$, (A/J X *M. Spretus*) $F_1$, and (C57BL/6J x BALB/cJ) $F_1$ mice (Table II) [35,49,50]. The chloramphenicol acetyltransferase (CAT) assay was used to compare the transcription-stimulating activity of K-*ras* intron 2 putative enhancer regions of $K^R$ and $K^S$ alleles using the nontumorigenic C10 cell line derived from normal alveolar Type II cells. This analysis demonstrated that enhancer activity of $K^S$ was 2.4–9.1-fold higher than that of $K^R$ and the *M. spretus* K-*ras* allele, irrespective of orientation [48, 51]. *Mus Spretus*, wild strain that is resistant to lung tumor formation, possesses a variant of K-*ras* with polymorphisms distinct from those of $K^S$, $K^I$, and $K^R$ alleles. Observed sequence variations in the intron 2 putative enhancer region among different strains of the K-*ras* gene may contribute to observed differences in the levels of K-*ras* expression among different mouse strains. These findings suggest that mutational activation of the more highly expressed K-*ras* allele may provide a selective

advantage during mouse hybrid lung carcinogenesis.

As shown in Table III, overexpression of c-*myc* proto-oncogene also has been observed at a high frequency in urethane-induced mouse lung tumors [12]. Northern blot analysis showed a 3–5-fold increase in c-*myc* transcripts in 8 of 11 (A/J X C3H/HeJ) $F_1$ and 4 of 5 BALB/c lung tumors. The c-*myc* proto-oncogene is an important regulator of cell proliferation and apoptosis, and its constitutive expression enforces proliferation and sensitizes cells to apoptosis [52]. Recent evidence suggests that c-*myc* appears to function as both activator and repressor of growth antagonistic genes [53]. Also, c-*myc* overexpression prevents growth arrest induced by p16 and can bypass p16/Rb enforced growth arrest. Thus, c-*myc* is thought to function downstream of p16/Rb, perhaps mimicking the effect of an inactive p16/Rb pathway [54]. Table III summarizes differentially expressed genes detected in mouse lung tumors.

Various other genes have shown increased levels of expression in mouse lung tumors. For example, Re, et al. reported a 3–5-fold increase in the mRNA level of the pulmonary surfactant protein-A (SP-A) gene in all tumors examined (11 (A/J X C3H/HeJ) $F_1$, 5 BALB/c, and 9 A/J) (Table III) [12]. The authors concluded that all tumors examined were derived from either alveolar type II or Clara cells on the basis that SP-A and other surfactants, including SP-B and SP-D, are selectively expressed by these cell types.

## TUMOR SUPPRESSOR GENES

Allelotype and LOH studies have been conducted to identify regions of frequent allelic loss in lung tumors of various $F_1$ hybrid mouse strains in order to localize important tumor suppressor genes [10,11,55–58]. As shown in Table IV, the loci most commonly affected by allelic loss were shown to reside on chromosome 4. Deletion mapping studies involving lung adenocarcinomas of (A/J x C3H/HeJ) $F_1$, (C3H/HeJ x A/J) $F_1$, (BALB/cJ x DBA/2J) $F_1$, and (C57BL/6J x C3H/HeJ) $F_1$ mice implicated two distinct lung tumor suppressor loci on this chromosome [10, 56–58]. One locus was mapped to the p15 and p16 genes on mid-chromosome 4, deletions of which occurred in about half of the adenocarcinomas examined [10]. The p15 and p16 genes were later shown to be homozygously codeleted in 12 of 16 (75%) lung tumor cell lines derived from inbred mouse strains [59]. Both of these studies demonstrated narrow regions of deletion, which strongly suggested that p16, and perhaps p15, inactivation contributes to mouse lung tumorigenesis. In comparison, human p16 and p15 have been the target of deletion at a similar frequency in human non-small cell carcinomas [60]. It is interesting that the p19ARF gene, which overlaps with p16, is also commonly deleted along with p16 in both human and mouse lung cancer, suggesting that this narrow region of the genome could harbor more than one lung tumor suppressor gene [61–64].

We recently demonstrated the existence of two variants of the mouse p16 gene [43]. Observed sequence polymorphisms constituted three amino acid differences; one at position 18 and another at position 51 of exon 2. Most strains encode a histidine (CAT) at position 18 and a valine (GTA) at position 51; however, six of the strains (BALB/c, O20, C3H/HeJ, C3H/21BG, CBA/J, and PL/J) code for proline (CCT) and isoleucine (ATA) at these positions, respectively. The p19ARF gene shares the p16 locus by utilizing the same second exon in an alter-

**TABLE III. Aberrant Levels of Gene Expression in Primary Mouse Lung Tumors***

| Gene | Alteration | | References |
|---|---|---|---|
| | mRNA | Protein | |
| Rb | Decreased | Decreased | [12, 65] (Liu et al., unpublished data) |
| p16 | Decreased | Decreased | [65] (Liu et al., unpublished data) |
| Growth arrest-specific 3 | Decreased | N.D. | [12] |
| Aldehyde dehydrogenase-I | Decreased | Decreased | [87] |
| Carbonic anhydrase-III | Decreased | Decreased | [88] |
| Carbonyl reductase | Decreased | Decreased | [88] |
| c-*myc* | Increased | N.D. | [12] |
| Surfactant protein A | Increased | N.D. | [12] |

*N.D., not determined.

**TABLE IV. Summary of Frequent Allelic Loss in Hybrid Mouse Lung Carcinomas***

| Chromosome | Identified target gene | Frequency (%) | Strain | Reference |
|---|---|---|---|---|
| 1 | None | 5/36 (14) | $AC3F_1$ | [57] |
| | | 5/15 (33) | $CDF_1$ | [57] |
| 4 | p16 | 6/16 (38) | $CDF_1$ | [10] |
| | | 18/36 (50) | $AC3F_1$ | [58] |
| | | 23/45 (51) | $C3AF_1$ | [10, 58] |
| | | 41/102 (40) | $B6C3F_1$ | [56] |
| | | 12/24 (50) | $AC3F_1$ | [56] |
| | | 11/24 (46) | $C3AF_1$ | [56] |
| 11 | p53 | 6/8 (75) | $B6C3F_1$ | [55] |
| | | 4/36 (11) | $AC3F_1$ | [57] |
| | | 19/72 (26) | $C3AF_1$ | [57] |
| 12 | None | 10/36 (28) | $AC3F_1$ | [57] |
| 14 | Rb | 1/8 (12) | $B6C3F_1$ | [55] |
| | | 10/36 (28) | $AC3F_1$ | [57] |
| | | 8/27 (29) | $C3AF_1$ | [57] |
| | | 4/15 (27) | $CDF_1$ | [57] |

*$AC3F_1$, (A/J × C3H/HeJ)$F_1$; $CDF_1$, (BALB/cJ × DBA/2J)$F_1$; $C3AF_1$, (C3H/HeJ × A/J)$F_1$; $B6C3F_1$, (C57BL/6J × C3H/HeJ)$F_1$.

nate reading frame; p16 and p19ARF have different first exons, referred to as exon 1α and exon 1β, respectively. The polymorphism resulting in a substitution at position 51 of p16 also produces a substitution at codon 72 of p19ARF. The two variants of p19ARF encode histidine or arginine at codon 72 with arginine co-segregating with H18 and V51. The above-mentioned LOH in lung carcinomas of intervariant F1 hybrids showed significant bias for loss of the allelic form coding for a H18 and V51, regardless of donor parent gender. For example, this allele was lost in 100% of (BALB/c x DBA/2J) F1, 84% of (C3H/HeJ x A/J) F1, 82% of (C57BL/6J x C3H/HeJ) F1, and 72% of the (A/J x C3H/HeJ) F1 lung tumors (biased allele lost is underlined). Studies using immunohistochemical analysis found that p16 inactivation is a frequent genetic defect in lung tumor progression [65] (Liu et al., unpublished data). Markedly reduced or absent p16 protein were affected in approximately half of the A/J and (C3H/HeJ x A/J) F1 lung adenocarcinomas, some of which revealed only focal areas of loss [65] (Liu et al., unpublished data). Many areas of loss were subsequently microdissected and shown by multiplex PCR analysis to display deletions of the p16 gene. Whereas hemizygous loss of the A/J allele was observed in 80% of the (C3H/HeJ x A/J) F1 tumors with no detectable p16 protein, 40% of the A/J tumors that showed an absence of p16 protein exhibited homozygous loss of the p16 gene. These results are consistent with the notion that the A/J allelic variant of p16 and/or p19ARF is a more potent growth/tumor suppressor. Our experiments have shown that transcriptional levels of the two mouse variants of the p16 and/or p19ARF do not differ significantly (Herzog et al., unpublished data). These data may suggest, therefore, that the C3H/HeJ variant of p16 and/or p19ARF exerts little selective pressure for its allelic loss in tumorigenesis. This possibility is currently being investigated.

A second region of LOH on mouse chromosome 4 was localized to distal microsatellite markers D4MIT54 and D4MIT158, syntenic to human chromosome 1p36, which is also commonly affected by LOH in multiple cancer types [58,66,67]. This region (about 3 cM) was affected by loss of heterozygosity in 44% of the lung adenocarcinomas tested. Either of the two loci on chromosome 4 underwent allelic loss in only 2% of the lung adenomas from (A/J x C57BL/6J)$F_1$, (C3H/HeJ x A/J) $F_1$, and (A/J x C3H/HeJ) $F_1$ mice [10,58]. These results suggested a role for the resident tumor suppressor loci in the progression or malignant conversion of mouse lung tumors.

Similarly, a pattern of LOH on chromosome 14 indicated that more than one tumor suppressor locus may reside on this chromosome [57]. Twenty-eight percent of the hybrid mouse lung adenocarcinomas displayed loss of heterozygosity on this chromosome. The Rb gene resides on chromosome 14 and has been an implied target for the LOH observed. However, a second region distantly centromeric to the Rb gene was found to undergo LOH at a frequency slightly greater than that observed for Rb [57]. This region of chromosome 14 has homology with the human chromosome 3p21–24, which is suspected to harbor a tumor suppressor gene based on frequent allelic loss in lung tumors [68].

That Rb inactivation plays a role in lung tumorigenesis has also been suggested by results showing a reduction in Rb expression in mouse lung adenomas and adenocarcinomas [12, 65] (Liu et al., unpublished results). For example, Rb mRNA expression was reduced 6–10-fold in all 25 of the (A/J x C3H/HeJ) $F_1$, A/J, and BALB/cJ lung adenomas examined in one study without detectable loss of heterozygos-

ity (Table III) [12]. Similarly, reduced Rb protein levels were observed in a high percentage of (C3H/HeJ x A/J) $F_1$ lung adenocarcinomas and A/J lung adenomas and adenocarcinomas using immunohistochemistry [65] (Liu et al., unpublished data). Interestingly, some of the adenocarcinomas that displayed Rb loss also showed focal loss of p16. In contrast to these findings, Western blot analysis of A/J adenocarcinomas showed little variation from the normal level of pRb expression, but significant variation between tumors in p16 protein levels [69]. Northern analysis also has shown somewhat higher levels of p16 mRNA in A/J lung adenomas and adenocarcinomas in comparison to normal surrounding tissue [65]. These analyses, therefore, may not be suited to detect focal aberrations. These results suggest that loss of Rb function may play a role early in mouse lung tumorigenesis, and that Rb and p16 inactivation occur together in lung adenocarcinomas. This observation is inconsistent with what has been reported in several human cancer types [70], including those of the lung, where this G1-S phase regulatory pathway is seen to function as a unit, with the alteration of one component precluding the selection for alterations in the remaining constituents [46]. However, unlike its human counterpart, mouse p16 appeared not to be influenced by Rb status in cultured fibroblasts [71]. A plausible scheme in mouse lung tumorigenesis may be that Rb down regulation occurs in lung adenomas and persists into malignancy, while loss of p16 occurs predominantly in adenocarcinomas, suggesting temporal specificity for inactivation of both in these tumors.

Recently, p53 germline mutations were examined in mouse lung susceptibility to methylnitrosourea [72]. p53 transgenic mice with a germline missense mutation (Ala135Val) were crossbred with A/J mice to study function of the p53 gene in mouse lung carcinogenesis. An average of 22 lung adenomas were observed in p53+/- mice and an average of 7 tumors in p53+/+ mice 16 weeks after exposure to methylnitrosourea, representing a 3-fold increase. However, this significant difference in tumor incidence was not seen when A/J mice were crossed with p53 knockout mice. These observations suggest that the mutant p53 allele increased lung tumor susceptibility.

## CHEMOPREVENTION STUDIES EMPLOYING THE MOUSE LUNG TUMOR MODEL

A growing understanding of the specific molecular changes during lung tumor progression makes it possible to elucidate the molecular mechanism(s) of lung cancer chemoprevention by certain agents, develop surrogate endpoint biomarkers for use in clinical lung cancer chemoprevention trials, and potentially develop new and effective chemopreventive agents by targeting key genetic changes detected during lung tumorigenesis. The lung tumor model has been used extensively for chemoprevention studies [72]. For example, phenethyl isothiocyanate was found to be an effective chemopreventive agent against 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK)-induced A/J mouse lung carcinogenesis [73]. As do most cancers, lung cancer develops over an extended period, apparently progressing sequentially from hyperplasia → dysplasia → adenoma → carcinoma → invasive cancer. The mouse lung tumor model progresses through many of these same stages. However, most chemopreventive studies in mice have employed development of adenomas as endpoints.

Wattenberg discussed two primary classes of chemopreventive agents based on their mechanisms of action—blocking agents and suppressing agents [75]. Most known carcinogens (e.g., aflatoxins, aromatic amines, nitrosamines, polycyclic hydrocarbons) need to be activated in the host to mutagenic and carcinogenic metabolites. Various mechanisms might effectively "block" the initial steps in carcinogenesis, e.g., altering the activities of various phase I enzymes (mostly cytochrome P450s) or phase II enzymes (conjugating enzymes), either altering production of mutagenic or carcinogenic moieties or increasing inactivation of genotoxic or carcinogenic moieties, directly blocking enzymes which activate the procarcinogens, or directly binding to or inactivating active carcinogenic or mutagenic moieties.

Most studies (see Table V) that identify chemopreventive agents in the mouse lung adenoma model have focused on the effects of these blocking agents. Several studies have reported on the inhibitory effects of isocyanates [72–75]. Other agents that block carcinogenic activation in the mouse lung adenoma assay include 5,6 benzoflavone, 2(3)-*tert*-butyl-hydroxyanisole (BHA), ethoxyquin, diallyl sulfide,

and sulindac (Table V) [76–79]. However, problems are associated with the typical chemopreventive studies employed to identify blocking agents in this model system. Most of these blockers show a marked preference for a particular class of carcinogen. For example, phenethylisothiocyanate (PEITC) is highly effective against various nitrosamine carcinogens (e.g., NNK, diethylnitrosamine) but highly ineffective against many other classes of carcinogens, e.g., polycyclic hydrocarbons. Some investigators have proposed the use of a combination of carcinogens, e.g., NNK and benzo(*a*)pyrene, which presumably may more accurately reflect the carcinogenic effects of cigarette smoke, the agent we are attempting to model. Also, most carcinogens are administered experimentally in large bolus doses, while environmentally most carcinogens are administered repeatedly at relatively low doses. This lower repeated dosing, more typical of environmental exposure to carcinogens, may make these compounds more susceptible to manipulation by blocking agents. One example of this is the finding of Pepin and coworkers [76] that sulindac is effective against NNK administered at low doses continually in drinking water, though totally ineffective against large bolus doses of the same compound. Most carcinogens are administered systemically, i.p. or i.g., in contrast to cigarette smoke, which is initially administered locally. This may lead to problems if a given blocking agent induces phase I and phase II enzymes in the liver and colon and thereby decreases the levels of carcinogen reaching the lung. Many blocking agents, particularly those that directly block the cytochromes that activate procarcinogens, have themselves been given as bolus doses shortly before the administration of carcinogen. Thus, it will be difficult to achieve a similar set of circumstances in humans. A final problem with chemopreventive studies is that the initiating agent employed is not cigarette smoke. Even though combinations of carcinogens may closely parallel it, cigarette smoke has literally hundreds of components with carcinogenic or promoting properties, making it more difficult to determine the immediate relevance of one or two carcinogenic agents.

Another type of chemopreventive agent is the suppressing agent. This term defines agents which act following the initiation stage of carcinogenesis. Chemopreventive agents with suppressive activities in the mouse lung adenoma assay include 2-difluromethylornithine (DFMO), perillyl alcohol, chalcones, myo-inositol, dexamethasone, budesonide, tea extract, lovastatin, and farnesol (Table V) [80–84] (Lubet et al., unpublished data). Given that continual exposure to mutagenic carcinogens (cigarette smoke) may occur even after initial dysplasia is achieved, it may be much more difficult to completely differentiate between suppression and blocking during progression of the disease in smokers. Nevertheless, agent(s) that are effective when given after a bolus dose of

**TABLE V. Agents That Have Chemopreventive Activity in the Mouse Lung Adenoma Assay***

| Chemical | Carcinogen employed | References |
|---|---|---|
| 5,6 benzoflavone | B(*a*)P, MCA | [77] |
| Ascorbic acid | B(*a*)P | [89] |
| b-naphthoflavone | B(*a*)P, MCA | [90, 91] |
| BHA | B(*a*)P, Urethane, DEN | [77] |
| Biochanin A | B(*a*)P | [92] |
| Black tea extracts | NNK, DEN | [80] |
| Budesonide | B(*a*)P | [93, 94] |
| Caffeine | B(*a*)P | [89] |
| Chalcones | | [81] |
| Dexamethasone | B(*a*)P, NNK | [82] |
| Dexamethasone + inositol | B(*a*)P, NNK | [82] |
| DFMO | B(*a*)P | (Lubet et al)** |
| Diallyl sulfide | B(*a*)P, NNK | [78–79] |
| D-limonene | NNK | [95] |
| Ellagic acid | B(*a*)P | [96] |
| Ethoxyquin | B(*a*)P, DEN | [77] |
| Farnesol | B(*a*)P | (Lubet et al)** |
| Ganoderma lucidum | B(*a*)P | [87] |
| Green tea extracts | NNK, DEN | [80] |
| Indole-3-carbinol | NNK | [97] |
| Lovastatin | B(*a*)P | [81] |
| Myo-inositol | B(*a*)P, NNK | [82] |
| PEITC | NNK | [74] |
| Perillyl alcohol | NNK | [84] |
| PHITC | NNK | [74] |
| Red ginseng extract | B(*a*)P | [89] |
| Sodium cyanate | B(*a*)P | [98] |
| Soybean lecithin | B(*a*)P | [89] |
| Sulindac | NNK | [76] |
| Tannic acid | B(*a*)P | [99] |

*BHA, 2(3)-*tert*-butyl-hydroxyanisole; DFMO, 2-difluromethylornithine; PEITC, phenethylisothiocyanate; PHITC, phenhexylisothiocyanate; B(*a*)P, benzo(*a*)pyrene; MCA, 3-methylcholanthrene; DEN, diethylnitrosamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.
** unpublished data.

carcinogen in the mouse lung adenoma model will be considered suppressing agents. This can vary from agents that cause cellular differentiation, to agents which may specifically alter enzymatic function in initiated cells, to agents that cause cell death or inhibit further growth in initiated cells. Chemopreventives can either be agents which apparently act on relatively early preinvasive lesions or agents which may work either early or late during the process of carcinogenesis (e.g., antiestrogens in breast cancer or cyclooxygenase inhibitors in colon).

Table V also summarizes a variety of agents which have been examined for chemopreventive activity in the mouse lung adenoma assay. One would expect that these agents would be equally effective against lesions induced by virtually any carcinogen. However, most of these agents have not been tested on lesions induced by different carcinogens. Perhaps the most effective suppressing agents used to date are tea extracts, which have inhibited adenoma multiplicity more than 65% in a number of studies. Certain of the more common classes of suppressor agents have not been routinely tested in the mouse lung adenoma model (e.g., vitamin D analogs, differentiating agents such as sodium butyrate, etc.). Nevertheless, a significant list of at least partially effective agents has been generated. This list is not meant to be inclusive but rather to give examples of agents which have been tested and may warrant further testing. Many chemopreventive agents which have shown efficacy in a variety of carcinogenesis models of other organs—e.g., DFMO (a specific inhibitor of ornithine decarboxylase), dexamethasone (glucocorticoid, anti-inflammatory), budesonide (a synthetic glucocorticoid), myo-inositol, green tea and black tea extracts—demonstrated significant efficacy in the mouse lung adenoma model. It may well prove that some combination of these agents will be particularly effective in the mouse lung tumor model.

## FUTURE PROSPECTS

Future avenues for exploration employing the mouse lung adenoma model should include combinations of chemopreventive agents, which may be significantly more effective than any one agent alone, e.g., myo-inositol + dexamethasone. Pulmonary administration of chemopreventive agents should be explored. Administration of certain agents by aerosol may decrease systemic toxicity and perhaps even increase efficacy. Preliminary studies have shown that PEITC and the glucocorticoid budesonide are highly effective when administered by this method. Agents for specific genetic lesions should be developed. As discussed in this article, perhaps the most common genetic alteration observed in mouse lung tumors is mutation in the K-*ras* gene. Since this gene must be isoprenylated to be active, chemicals that specifically block this reaction may show activity against development of these tumors. Part of the activity of both lovastatin and farnesol (Table V) is likely related to their ability to alter isoprenylation. Preliminary studies employing highly specific inhibitors of farnesyltransferase or geranylgeranyltransferase appear to exhibit activity in this specific tumor model. Although we have briefly discussed protocols related to K-*ras*, other possible gene targets (RB, p16, etc.) are frequently altered and may be useful for chemoprevention. The mouse lung adenoma assay, with its routine alterations in certain oncogenes and tumor suppressor genes, may prove particularly applicable to studies in antisense gene therapy. Development of a cigarette smoke–induced mouse model of lung tumorigenesis would appear to be of great potential use, particularly when looking for agents that block tumorigenesis. Further identification of mouse lung susceptibility genes and characterization of transgenic models of human cancer genes will provide more appropriate animal models for familial lung cancer in humans. And finally, a systematic analysis of genetic alterations and differentially expressed genes in mouse lung tumors treated with and without known chemopreventive agents will contribute significantly to the development of surrogate endpoint biomarkers for economical and efficient clinical testing of prospective chemopreventive agents.

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