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Genetic Alterations in *K-ras* and *p53* Cancer Genes in Lung Neoplasms From B6C3F1 Mice Exposed to Cumene

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Abstract

The incidences of alveolar/bronchiolar adenomas and carcinomas in cumene-treated B6C3F1 mice were significantly greater than those of the controls. We evaluated these lung neoplasms for point mutations in the *K-ras* and *p53* genes that are often mutated in humans. *K-ras* and *p53* mutations were detected by cycle sequencing of PCR-amplified DNA isolated from paraffin-embedded neoplasms. *K-ras* mutations were detected in 87 % cumene-induced lung neoplasms, and the predominant mutations were exon 1 codon 12 G to T transversions and exon 2 codon 61 A to G transitions. *P53* protein expression was detected by immunohistochemistry in 56 % cumene-induced neoplasms and mutations were detected in 52 % neoplasms. The predominant mutations were exon 5, codon 155 G to A transitions and codon 133 C to T transitions. No *p53* mutation and one of 7 (14 %) *K-ras* mutation was detected in spontaneous neoplasms. Cumene-induced lung carcinomas showed loss of heterozygosity (LOH) on chromosome 4 near the *p16* gene (13 %) and on chromosome 6 near the *K-ras* gene (12 %). No LOH was observed in spontaneous carcinomas or normal lung tissues examined. The pattern of mutations identified in the lung tumors suggests that DNA damage and genomic instability may be contributing factors to the mutation profile and development of lung cancer in mice exposed to cumene.

Keywords

K-ras; *p53*; cumene; lung; mouse

Introduction

Lung cancer is the most frequently diagnosed 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. 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.

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Mouse alveolar/bronchiolar adenomas and carcinomas, which are common spontaneous and chemical induced lung tumors in mice, are similar to human adenocarcinomas in histomorphology and molecular characteristics including activation of the *K-ras* gene (25, 27). The patterns of mutations in cancer genes, such as *ras* and *p53,* have been found to aid in the understanding of tumorigenesis in rodents and humans (7,21,24,28). For example, in some neoplasms, the profile of activating mutations in *ras* genes or inactivating mutations in the *p53* gene are specific for particular chemicals and differ from those detected in spontaneous neoplasms (38,39).

Cumene, or isopropylbenzene, is a constituent of crude oil used primarily for the production of phenol and acetone. It is a good solvent for fats and resins and has been suggested as a replacement for benzene in many industrial applications. The annual production of cumene in the US is high and increasing with 4.49 billion pounds produced in 1993 (9). Being a major commodity chemical, there is a high potential for many workers to be exposed to cumene. The most probable route of human exposure is inhalation of contaminated air (16) from cumene evaporated into the environment during production and processing from petroleum refining, combustion of petroleum products, and use of a variety of products containing cumene.

National Toxicology Program (NTP) 2-year whole-body inhalation studies revealed that treatment of B6C3F1 mice with cumene significantly increased the incidence of alveolar/bronchiolar adenomas and carcinomas in all groups of exposed males and females. Cumene is not genotoxic which was demonstrated by several studies involving bacterial and mammalian cells in culture and in *in vivo* studies involving mice and rats (10,26,43). *In vitro* cell transformation assays using BALB/3T3 mouse embryo cells and unscheduled DNA synthesis assays using rat primary hepatocytes yielded conflicting results regarding a cumene effect that were not reproducible. Cumene was weakly positive with no clear dose response for the induction of micronuclei in rat bone marrow at doses ranging from 78 to 2500 mg/kg intraperitoneally (26).

The goal of this study was to evaluate both spontaneous and cumene-induced lung neoplasms for mutations in the *K-ras* and *p53* genes, which are considered important in the pathogenesis of human lung cancer. Another goal was to identify chemical specific mutations that might serve as biomarkers of occupational exposure with possible relevance to human health.

Materials and Methods

Lung Neoplasms

Male and female B6C3F1 mice were exposed to 0, 125 (females only), 250, 500, or 1000 (males only) ppm cumene (50 animals each group) by whole-body inhalation, 6hr/day, 5 days/week for 2 years (NTP, Cas Number 98-82-8). Husbandry and experimental procedures were in compliance with the requirements set forth by the Public Health Service's *Guide for the Care and Use of Laboratory Animals.* Incidences of cumene-induced lung tumors were evaluated statistically using the poly-3 test. At necropsy, tissues were fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, sectioned to a thickness of 5 *u*m, and stained with hematoxylin and eosin. Subsequently, 5 unstained serial sections, 10 *u*m thick, were prepared from paraffin blocks containing alveolar/bronchiolar adenomas or carcinomas. In order to isolate adequate amounts of DNA, neoplasms greater than 1 mm in diameter were identified for analysis. Fifty- two cumene-induced alveolar/bronchiolar neoplasms (6 adenomas and 46 carcinomas), 7 spontaneously occurring carcinomas, and 6 normal lung tissues were evaluated for *K-ras* mutations in exons 1 and 2 (codons 12, 13 and 61) and *p53* mutations in exons 5–8.

DNA Isolation, Amplification, and Cycle Sequencing

DNA was isolated and extraced from paraffin-embedded sections containing lung neoplasms and normal lung tissues and amplified by PCR. Details of the use of nested primers for *K-ras* and *p53* genes have been described previously (20,37). Positive DNA controls for *K-ras* and *p53* mutations and controls lacking DNA were run with all sets of reactions. PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified samples were sequenced utilizing a cycle sequencing kit (US Biochemical, Cleveland, OH), which incorporated [α-^{33}p]-dideoxynucleotide triphosphate (ddNTP) terminators (A, C, G, T) into the sequencing products. Detected mutations were confirmed by repeat analysis, starting from amplification of the original DNA extract.

Immunohistochemistry for p53 Protein

Alveolar/bronchiolar adenomas and carcinomas were examined for *p53* protein expression by immunohistochemical analysis, using an avidin-biotin-peroxidase detection system (Vectastain Rabbit Elite Kit, Vector Laboratory, Burlingame, CA). The immunohistochemical staining for expression of mutant *p53* protein was performed as previously described (12,13, 15,23,39). A 1:300 dilution of the primary polyclonal rabbit antibody CM-5 (Vector Laboratories), which detects accumulation of the mutant *p53* in rodents, was used. Normal rabbit serum (Vector Laboratories) was used as the negative control in place of the primary antibody. Tissue specimens from a *p53* transgenic mouse (mutation in codon 135 of *p53)* served as the positive control.

Loss of Heterozygosity (LOH) Analysis

Microsatellite marker Mts 1 near the *p16* tumor suppressor gene on chromosome 4 was used to amplify polymorphic regions between strain C57BL/6 (B) and C3H/He (H) mice (Jackson Laboratories, Bar Harbor, Maine) to identify portions of tumors with LOH at the *p16* locus. The Mts 1 forward (5′-GA TTT CTA CGG AAA GCC CTG-3′) and reverse (5′-TAT TGT GCA TTT GTG TGT CTG G-3′) primers were located 2395 and 2172 bp upstream of the translational start site, respectively. PCR amplification was performed on 40 cumene-induced lung neoplasms (36 carcinomas, 4 adenomas) and 7 spontaneous B6C3F1 carcinomas. Controls lacking DNA were included with all amplifications. The PCR products were resolved on 4% NuSieve (FMC BioProducts, Rockland, ME.)- agarose (3:1) to separate B and H alleles.

The inner PCR products from paraffin-embedded plus 8 frozen treated neoplasms (7 carcinomas & 1 adenoma) were labeled by incorporation with [α-^{33}p]-dATP (MP/ICN Biomedicals, rvine, CA), using single stranded conformation polymorphism (SSCP) analysis to distinguish between the H and B alleles on a 0.5X MDE (Cambrex Bio Science, Rockland, ME) gel at 3W for 15 hours at room temperature.

To determine LOH in 50 cumene-induced lung neoplasms (46 carcinomas, 4 adenomas) and 7 spontaneous carcinomas at marker D6MCO12 near the *K-ras* gene on chromosome 6 in B6C3F1 mice, SSCP analysis was utilized. PCR amplification with [α-^{33}p] - labeled and unlabeled dATP was performed as described previously (39). The MDE gel was electrophoresed at 3W at room temperature for 15 hours to separate the H and B alleles. The gels were dried and exposed to X-ray film overnight.

Results

Mutation Analysis

The incidence of alveolar/bronchiolar adenomas and carcinomas in male and female treated groups was significantly greater than those of the controls (Table 1). A higher frequency of *K-ras* mutations (45/52, 87 %) was observed in the cumene-induced lung neoplasms, as

compared to spontaneous lung neoplasms from control animals (historical 33/117, 28 %; concurrent 1/7, 14 %) (Table 2). These mutations were observed in 91% (41/45) of males and 57% (4/7) of females. *K-ras* mutations were detected in 67% (4/6) of the adenomas (1 in codon 13 and 3 in codon 61) and in 89% (41/46) of the carcinomas. The predominant *K-ras* mutations were codon 12 GTT (G to T transversions) and codon 61 CGA (A to G transitions). Three codon 12 CGT mutations and one codon 61 CTA mutation were found in the cumene-induced neoplasms, but none were found in spontaneous lung neoplasms (0/124, concurrent controls combined with historical controls) (Table 2; Fig. 1a & 2). There was no significant increase in the incidence of mutations at codon 13 in the cumene-induced lung neoplasms.

p53 protein expression was detected by immunohistochemistry in 29/52 (56%) of cumene-induced lung neoplasms (Fig. 3), and mutations were identified in 27/52 (52%) of the neoplasms. *p53* protein expression and gene mutation were not detected in the 7 spontaneous carcinomas or in the 6 normal lung tissues except one spontaneous carcinoma with positive *p53* protein expression (Table 3). The predominant *p53* mutations were identified in exon 5 (24/27, 89 %) and in exon 7 (3/27, 11%) (Table 3; Fig. 4). No mutation was detected at exons 6 and 8 in the samples examined.

The *p53* mutations were observed in 58% (26/45) of males and 14% (1/7) of females. Mutations were detected in 50% (3/6) of the adenomas and 52% (24/46) of the carcinomas. There was a dose-dependent increase in incidence of *K-ras* and *p53* mutations, however, a similar mutation spectrum of both mutations was detected in cumene-induced neoplasms regardless of whether the neoplasms were adenomas or carcinomas.

LOH Analysis

LOH on chromosome 6 near *K-ras* gene was observed in 6/50 (12 %; 4 –H & 2 –B) treated carcinomas, 0/6 of adenomas, and 0/7 of the spontaneous carcinomas examined (Fig. 1b).

LOH of the C3H/He allele (H) was observed in 5/40 (13 %) cumene-induced carcinomas, 1/6 of adenomas, and 0/7 of the spontaneous carcinomas examined at the microsatellite marker on chromosome 4 near the *p16* gene (Fig. 5).

Discussion

There was a high frequency (87%) of *K-ras* mutations in cumene-induced alveolar/bronchiolar neoplasms compared to that in spontaneous alveolar/bronchiolar neoplasms from untreated B6C3F1 mice (28% historical database; 14% concurrent controls). The predominant mutations were *K-ras* codon 12 G to T transversions (GGT to GTT, 21%) and codon 61 A to G transitions (CAA to CGA, 25%), which clearly differed from those identified in untreated control mice (0.008% & 2%, respectively). Point mutations at codon 12 of the *K-ras* gene are activating mutations, rendering *ras* insensitive to the down-regulatory action of GTPase activating proteins, thereby locking the protein in the active state and promoting cellular transformation (2).

The high frequency and pattern of *K-ras* mutations in mouse lung tumors may directly depend on the nature of the chemical carcinogen or its metabolites. In our study, the development of lung neoplasms in B6C3F1 mice exposed to cumene may involve multiple carcinogenic processes including direct DNA damage and/or indirect DNA damage. Metabolites of cumene may have caused DNA adducts and subsequent point mutations. Side-chain oxidation of cumene (isopropylbenzene) is rapid and extensive and occurs in both hepatic and extra-hepatic tissues including the lung (35), with the secondary alcohol 2-phenyl-2-propanol being the principal metabolite in rats (30,43) and humans (25,43). The C-isopropyl bonds are readily cleaved, and the remaining electrophilic carbon moiety may form DNA adducts and cause

subsequent DNA damage. Previous studies showed that the related benzene is carcinogenic and genotoxic (1,6,40,44).

Alternatively indirect damage from oxidative stress may have contributed to the mutations. G to T transversions are commonly detected DNA base changes associated with active oxygen species and are consistent with 8-OH-G adducts produced during oxidative damage to DNA (17,18,36,42). In other studies, exposure of B6C3F1 mice to ozone or vanadium pentoxide is thought to result in the generation of hydroxyl radicals which induce a G to T transversion at codon 12 of *K-ras* gene (3,37). Interestingly, G to T transversion in *K-ras* codon 12 is the most common mutation detected in human adenocarcinomas (32). In human lung tumors, *K-ras* mutations appear to correlate with DNA adducts of benzo(a)pyrene and are associated with smoking (34). It is possible that smoking in combination with cumene exposure in humans may have an additive effect on *K-ras* mutations.

While *K-ras* appears to play a critical role in mouse and human lung adenocarcinoma formation, its function is complex. Zhang et al. (46) proposed that wild-type *K-ras* may be a mouse lung tumor suppressor gene. Further LOH analysis in cumene-induced lung neoplasms demonstrsted allelic loss on chromosome 6 near the *K-ras* gene in 6 carcinomas and on chromosome 4 near *p16* tumor suppressor gene in 5 carcinomas. Allele loss of *p16* has been detected in human cancers (4,19) and mouse lung tumors (29).

A high frequency (52%) of *p53* mutations were detected in cumene-induced alveolar/bronchiolar neoplasms which were correlated with increased *p53* protein expression (56%) by immunohistochemistry. The presence of *p53* protein expression without *p53* gene mutation could be due to mutations outside exons 5–8 or possibly due to alterations of other proteins downstream of *p53* (5). The predominance of cumene-induced alveolar/bronchiolar neoplasms that contain *p53* mutations may provide a selective advantage for unregulated growth and the avoidance of apoptosis (5,8,28,33). A study of aflatoxin-B1 (AFB1)-induced mouse lung tumors found a high proportion (>70%) of tumors with *p53* accumulation and mutations (41), and lung tumors of mice exposed transplacentally to AZT also had a high proportion (84 %) of *p53* mutations. (14). Other studies such as methylene chloride-induced mouse lung tumors (11), ozone-induced, and vanadium pentoxide-induced mouse lung tumors (3,37) exhibited no or low frequency of *p53* mutations. Unlike the mostly random mutation pattern for the AFB1-induced tumors (41), the cumene-induced tumors displayed specific *p53* mutations. *P53* mutations were only observed in exon 5 (24/27, 89%) and exon 7 (3/27, 11%). The mutations observed in the *p53* gene in cumene-exposed mice clearly imply this genetic event is related to chemical exposure, since these mutations were not detected in spontaneous tumors.

In conclusion, the patterns of *K-ras* and *p53* mutations identified in the cumene-induced lung tumors suggest that DNA damage and genomic instability may be the contributing factors to the mutation profile and development of lung cancer in these mice. The molecular alterations identified in the cumene-induced lung neoplasms appear to affect the same pathways as those reported in human lung cancer, suggesting that the response in the mouse may be of relevance to humans. Futher research of the cumene mouse lung tumors by microarray analysis underscore the complexity of lung cancer where in addition to genetic factors, signal transduction pathways and epigenetic mechanisms are also contributing factors to the carcinogenesis process (45).

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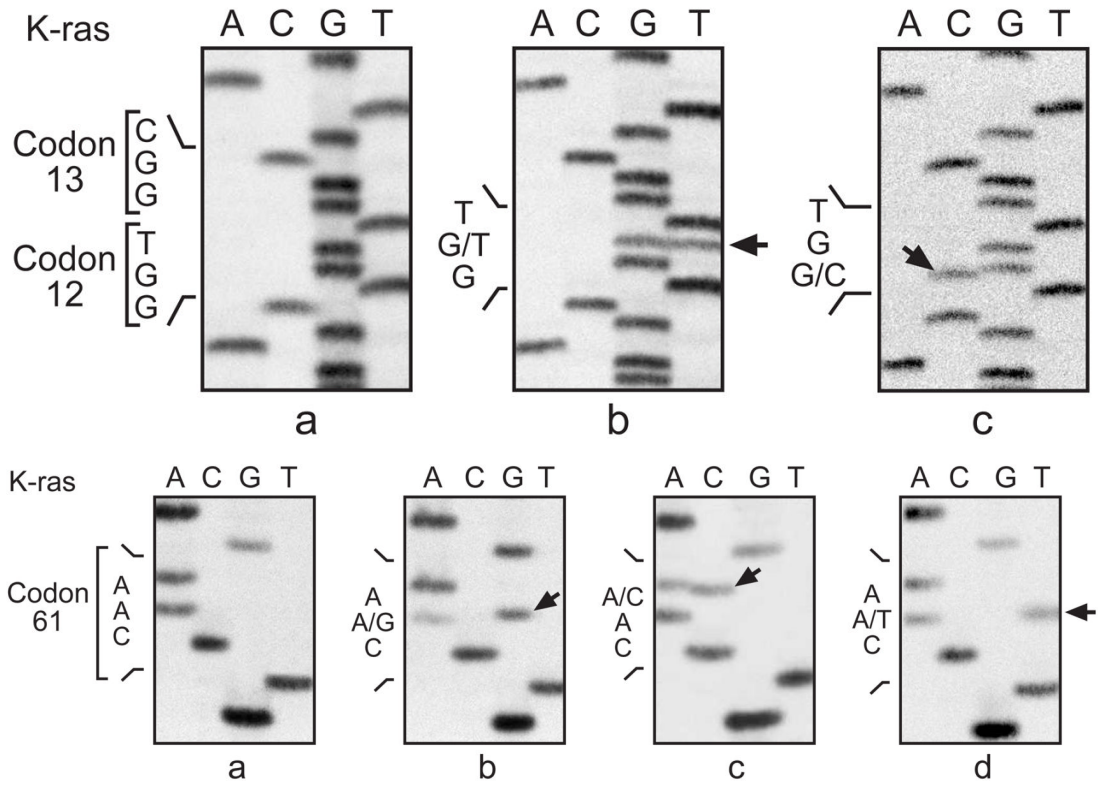

Fig. 1.

Fig. 1a. Identification of *K-ras* mutations in codon 12 of lung neoplasms from B6C3F1 mice exposed to cumene by whole-body inhalation for 2 years. Sequencing panels are from left to right: (a) normal *K-ras* codon 12 sequence GGT: (b) mutated codon 12 sequence GTT from animal No. 819 carcinoma; (c) mutated codon 12 sequence CGT from animal No. 808 carcinoma. Arrows point to mutant bands.

Fig. 1b. SSCP analysis at marker D6MCO12 to assess LOH on chromosome 6 near *K-ras* gene in cumene-induced carcinomas. Lanes 3–5 represent normal (N) lungs from B6C3F1 (F1), C3H (H) and C57BL/6 (B) inbred mouse strains. Lane 2 (animal No. 644 carcinoma) showed loss of C3H (H) allele: lane 6 (animal No. 846 carcinoma) showed loss of C57 (B) allele; lane 1 (animal No. 828 carcinoma) appeared normal with both alleles present.

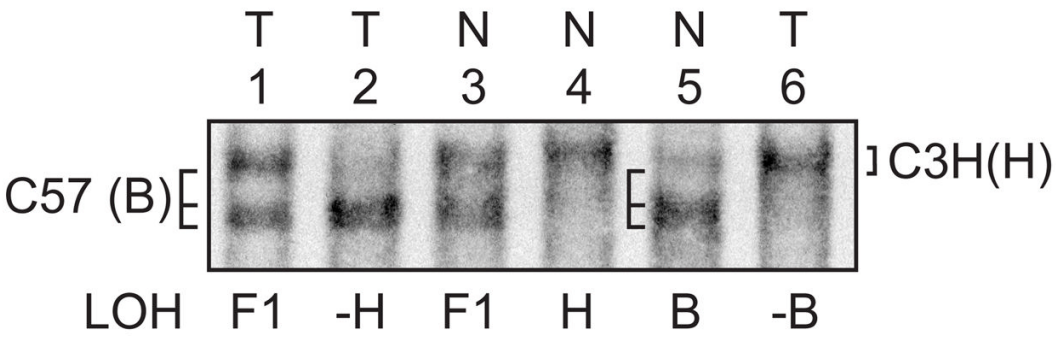

Fig. 2.
Identification of *K-ras* codon 61 mutations in cumene-induced lung neoplasms from B6C3F1 mice by cycle sequencing of amplified exon 2. Sequencing panels are from left to right: (a) normal codon 61 sequence CAA; (b) mutated sequence CGA from animal No. 604 adenoma; (c) mutated sequence CAC from animal No. 430 carcinoma; (d) mutated sequence CTA from animal No. 824 carcinoma. Arrows point to mutant bands.

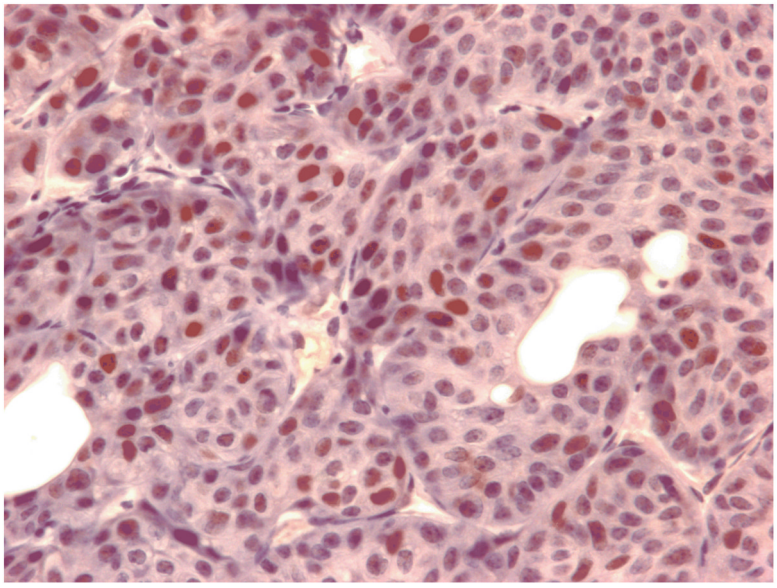

Fig. 3.
Alveolar/bronchiolar carcinoma from a male mouse exposed to 1000 ppm cumene. Note *p53* protein expression (brown chromogen) in the nucleus of malignant epithelial cell, 400x.

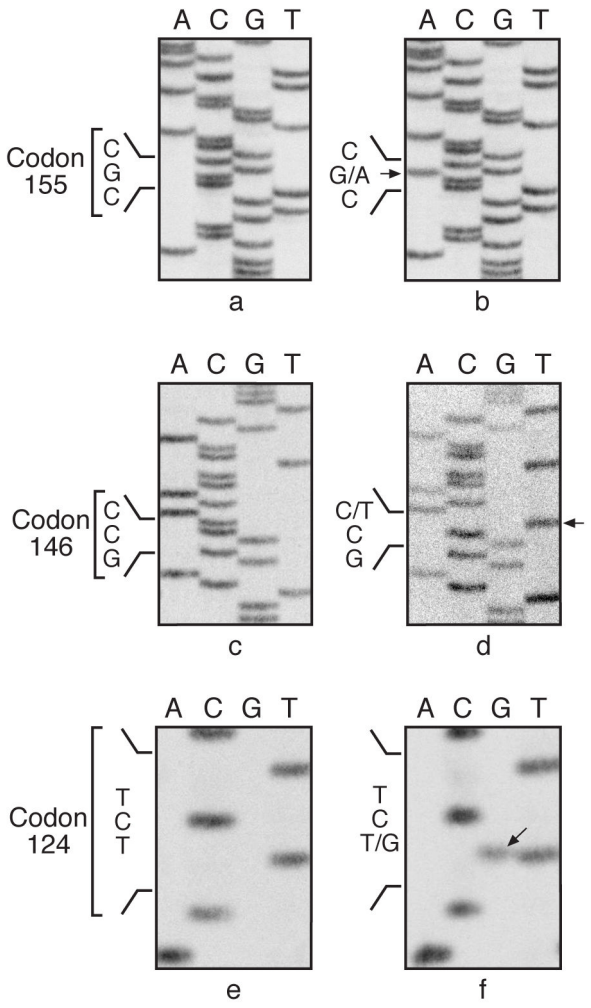

Fig. 4.

Examples of sequencing *p53* exon 5 mutations in lung neoplasms from B6C3F1 mice exposed to cumene. Sequencing panels a, c and e are normal *p53* sequences and panels b, d and f are mutated sequences at indicated codons from animal No. 402 carcinoma, animal No. 840 carcinoma, and animal No. 601 carcinoma. The wild-type allele was not detected in the sequencing reaction shown in panel (d).

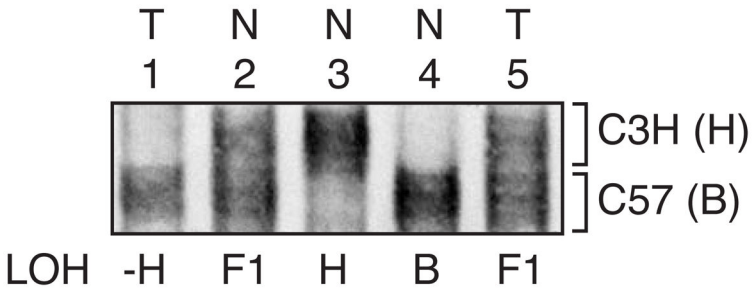

Fig. 5.
SSCP analysis at Mts 1 to assess LOH on chromosome 4 near *p16* tumor suppressor gene in cumene-induced carcinomas. Lanes 2–4 represent normal (N) lungs from B6C3F1 (F1), C3H (H) and C57 (B) inbred mouse strains. Lane 1 (animal No. 324 carcinoma) showed loss of the C3H (H) allele; lane 5 (animal No. 834 carcinoma) appeared normal with both alleles present.

Table 1

Incidences of Cumene-lnduced Lung Tumors in a 2-Year Inhalation Study of B6C3F1 Mice[a]

Lung Tumors	Males				Females			
	0 ppm	250 ppm	500 ppm	1000 ppm	0 ppm	125 ppm	250 ppm	500 ppm
Alveolar/Bronchiolar Adenoma	13/50 (26%)	31/50* (62%)	31/50* (62%)	29/50* (58%)	1/50 (2%)	26/50* (52%)	36/50* (72%)	38/50* (76%)
Alveolar/Bronchiolar Carcinoma	9/50 (18%)	19/50* (38%)	32/50* (64%)	33/50* (66%)	3/50 (6%)	16/50* (32%)	20/50* (40%)	34/50* (68%)
Alveolar/Bronchiolar Adenoma or Carcinoma	19/50 (38%)	38/50* (76%)	42/50* (84%)	43/50* (86%)	4/50 (8%)	31/50* (62%)	42/50* (84%)	46/50* (92%)

[a]Male and female B6C3F1 mice were exposed to 0, 125 (females only), 250, 500, or 1000 (males only) ppm cumene by whole-body inhalation, 6hr./day, 5 days/week for 2 years (NTP TR 542, 2007).

*Significantly different ($p<0.001$) from the control group by the poly-3 test.

Table 2

K-*ras* Mutations in Lung Neoplasms of B6C3F1 Mice in a 2-Year Inhalation Study of Cumene

Treatment ppm	Activate K-*ras*(%)	Codon12(GGT)	(GAT)	(TGT)	(GTT)	(CGT)	Codon13(GGC)	(CGC)	Codon 61 (CAA)	(CGA)	(CAT)	(CAC)	(CTA)
Control													
Historical[a]	33/117 (28)	14	5	1	0		6		2	4	1	0	
Concurrent	1/7 (14)	0	0	0	0		0		1	0	0	0	
Cumene[b]	45/52 (87)	6	5	11	3		4		13	0	2	1	
125	1/4 (25)	0	1	0	0		0		0	0	0	0	
250	10/13 (77)	0	0	1	2		0		5	0	2	0	
500	17/18 (94)	4	1	6	0		2		4	0	0	0	
1000	17/17 (100)	2	3	4	1		2		4	0	0	1	

[a] historical spontaneous lung neoplasms from control B6C3F1 mice (Hong et al., 2007).

[b] Male and female B6C3F1 mice were exposed to 0, 125 (females only), 250, 500, or 1000 (males only) ppm cumene by whole-body inhalation, 6hr./day, 5 days/week for 2 years.

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Table 3

p53 Mutations in Lung Neoplasms of B6C3F1 Mice in a 2-Year Inhalation Study of Cumene[a]

Treatment ppm	Activate *p53*[b](%)	IHC Positive No. (%)	Exon5	Exon7
Control	0/7 (0)	1/7 (14)	0	0
Cumene	27/52 (52)	29/52 (56)	24	3
125	0/4 (0)	1/4 (25)	0	0
250	5/13 (38)	6/13 (46)	4	1
500	11/18 (38)	8/18 (44)	10	1
1000	11/17 (65)	14/17 (82)	10	1

[a] Male and female B6C3F1 mice were exposed to 0, 125 (females only), 250, 500, or 1000 (males only) ppm cumene by whole-body inhalation, 6hr./day, 5 days/week for 2 years.

[b] No mutation detected for *p53* at exons 6 & 8 in the samples examined.