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K-*ras* Mutations in Lung Tumors and Tumors from Other Organs are Consistent with a Common Mechanism of Ethylene Oxide Tumorigenesis in the B6C3F1 Mouse

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Abstract

Ethylene oxide is a multisite carcinogen in rodents and classified as a human carcinogen by the National Toxicology Program. In 2-year mouse studies, ethylene oxide (EO) induced lung, Harderian gland (HG), and uterine neoplasms. We evaluated representative EO-induced and equivalent spontaneous neoplasms for K-*ras* mutations in codons 12, 13, and 61. K-*ras* mutations were identified in 100% (23/23) of the EO-induced lung neoplasms and 25% (27/108) of the spontaneous lung neoplasms. Codon 12 G to T transversions were common in EO-induced lung neoplasms (21/23) but infrequent in spontaneous lung neoplasms (1/108). K-*ras* mutations were found in 86% (18/21) of the EO-induced HG neoplasms and 7% (2/27) of the spontaneous HG neoplasms. Codon 13 G to C and codon 12 G to T transversions were predominant in the EO-induced HG neoplasms but absent in spontaneous HG neoplasms (0/27). K-*ras* mutations occurred in 83% (5/6) of the EO-induced uterine carcinomas and all were codon 13 C to T transitions. These data show a strong predilection for development of K-*ras* mutations in EO-induced lung, Harderian gland, and uterine neoplasms. This suggests that EO specifically targets the K-*ras* gene in multiple tissue types and that this event is a critical component of EO-induced tumorigenesis.

Keywords

K-*ras*; ethylene oxide; mice; carcinogen; Harderian gland; lung; uterus; neoplasm

Introduction

Ethylene oxide (EO) is a major industrial chemical used primarily as an intermediate in the manufacture of several industrial chemicals. Exposure to EO is greatest in the health care industry where EO is used as a sterilizing agent and an estimated 75,000 workers are potentially exposed (NTP, 1987, 2004; IARC, 1994; Recio et al., 2004). In spite of its widespread application, EO is harmful to human health and is considered a human carcinogen (Steenland et al., 1991; Lerda and Rizzi, 1992; Shore et al., 1993; Hengstler et al., 1994; IARC, 1994; Nygren et al., 1994). In vitro and in vivo data in rodents support its role as a mutagen and carcinogen (Shore et al., 1993; Hengstler et al., 1994; Nygren et al., 1994; Lorenti Garcia et al., 2001). National Toxicology Program (NTP) studies revealed that EO is a multisite carcinogen in rodents capable of inducing neoplasia in the lung, Harderian gland (HG) and uterus (NTP, 1987). The primary objective of this study was to evaluate representative

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examples of these EO-induced neoplasms as well as equivalent spontaneous neoplasms for genetic alterations in the major cancer gene K-*ras*. The goal was to help define potential mechanisms underlying EO-induced tumorigenesis and to identify chemical specific mutations that might serve as biomarkers of occupational exposure with possible relevance to human health.

Materials and Methods

Lung, Harderian Gland, and Uterine Neoplasms

Male and female B6C3F1 mice were exposed to 0, 50, or 100 ppm ethylene oxide (50 animals each group) by inhalation 6 hours/day, 5 days/week for 2 years (NTP, 1987). 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.* At necropsy, tissues were fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, sectioned to a thickness of 5 μm and stained with hematoxylin and eosin. Subsequently, 5 unstained serial sections, 10 μm thick, were prepared from paraffin blocks containing alveolar/bronchiolar adenomas or carcinomas, Harderian gland adenomas, or carcinomas or uterine carcinomas. In order to isolate adequate amounts of DNA, neoplasms greater than 1 mm in diameter were identified for analysis. A total of 23 lung neoplasms (5 alveolar/bronchiolar adenomas and 18 alveolar/bronchiolar carcinomas) and 108 spontaneous lung neoplasms (8 were concurrent controls) were analyzed for K-*ras* mutations in exons 1 and 2 (codons 12, 13 and 61). Twenty-one ethylene oxide-induced HG neoplasms (20 cystadenomas and 1 cystadenocarcinoma) and 27 spontaneous HG neoplasms (2 were concurrent controls) were evaluated for K-*ras* mutations in exons 1 and 2 (codons 12, 13 and 61). Six EO-induced uterine carcinomas were examined for K-*ras* mutations in exon 1 (codons 12 and 13). There were no concurrent uterine carcinomas in control mice.

DNA Isolation, Amplification, and Cycle Sequencing

DNA was isolated and extracted from paraffin-embedded sections containing lung, HG, and uterine neoplasms and was amplified by the polymerase chain reaction (PCR). Details of the nested primers for the K-*ras* gene have been described previously (Devereux et al., 1991, 1993; Sills et al., 1995). A positive control for K-*ras* and a control without DNA (distilled water) 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 (U.S. Biochemical, Cleveland, OH), which incorporates α-^{33}P dideoxynucleotide (ddNTP) terminators (A, C, G, T) into the sequencing products. Mutations were confirmed by repeated sequencing starting from amplification of the original DNA extract.

Results

Lung Neoplasms

Two years of inhalation exposure to EO caused lung neoplasms in B6C3F1 mice (Table 1). Twenty-three of these lung neoplasms from EO-exposed B6C3F1 mice and 108 lung neoplasms from untreated B6C3F1 controls (8 concurrent controls and 100 historical controls) were examined for mutations in exons 1 and 2 of the K-*ras* gene. A high frequency of K-*ras* mutations (23/23, 100%) was observed in the EO-induced lung neoplasms, as compared to spontaneous lung neoplasms from control animals (27/108, 25%) (Table 2). Mutations in spontaneous neoplasms included just 1 of 8 (13%) concurrent controls and 26 of 100 (26%) historical controls. Codon 12 mutations were the most common in both control and treated animals although the EO-induced neoplasms predominantly exhibited GGT to GTT mutations (21/23, 91%) as compared to the more common GGT to GAT mutations (11/27, 41%) in

spontaneous neoplasms (Figure 1). Codon 12 GGT to GTT were infrequent in spontaneous lung neoplasms (1/108, 0.9%). Codon 61 mutations were more common in spontaneous lung neoplasms occurring in 7 of 108 (7%) controls as opposed to only 1 of 23 (4%) EO-induced neoplasms. A similar spectrum of K-*ras* mutations was detected in EO-induced neoplasms regardless of histo-logic subtype (adenoma or carcinoma) or dose group; however, there was a slight dose-related increase in incidence of K-*ras* mutations (Table 2). Two mice from the 100-ppm dose group exhibited double mutations. One had a GGT to GTT mutation at codon 12 and a GGC to AGC mutation at codon 13 and the other had a GGT to GAT mutation at codon 12 and a CAA to CGA mutation at codon 61.

Harderian Gland Neoplasms

The incidence of EO-induced Harderian gland (HG) neoplasms is summarized in Table 1. Twenty-one HG neoplasms from EO exposed B6C3F1 mice and 27 spontaneous HG neoplasms from control B6C3F1 mice (2 concurrent controls and 25 historical controls) were examined for mutations in exons 1 and 2 of the K-*ras* gene. A high frequency (18/21, 86%) of K-*ras* mutations was detected in EO-induced HG neoplasms as compared to spontaneous HG neoplasms (2/27, 7%). Mutations in spontaneous neoplasms included just 2 of the 25 historical controls and none of the 2 concurrent controls. The predominant mutations in EO-induced HG neoplasms consisted of GGC to CGC transversions at codon 13 (15/18, 83%) and GGT to TGT transversions at codon 12 (8/18, 44%). Neither of these mutations was found in spontaneous HG neoplasms (0/27) (Table 3; Figure 2). A similar incidence and spectrum of K-*ras* mutations were detected in the 50-ppm and 100-ppm dose groups (Table 3). Point mutations were not detected in DNA isolated from 4 nontumor regions of the Harderian gland from control or EO-exposed mice. Eight of 11 mice (73%) in the 50-ppm dose group and 4 of 10 mice (40%) in the 100-ppm dose group had 2 or more mutations per neoplasm.

Uterine Neoplasms

The incidence of EO-induced uterine carcinomas is summarized in Table 1. All 6 uterine carcinomas from EO-exposed B6C3F1 mice were examined for mutations in exon 1 of the K-*ras* gene. The predominant mutation was a GGC to GGT transition in codon 13 (5/6, 83%) (Table 4 and Figure 2).

Discussion

To our knowledge, this is the first study to evaluate EO-induced neoplasms for genetic alterations in K-*ras*. Here we show that K-*ras* mutations are particularly common in EO-induced lung, Harderian gland, and uterine neoplasms suggesting that this is a critical event underlying the mechanism behind EO-induced tumorigenesis.

EO is a direct-acting carcinogen that reacts with nucleophilic molecules to form a variety of different adducts involving DNA, RNA and protein (Brown et al., 1996). It is a powerful mutagen and clastogen at all phylogenetic levels capable of inducing an increased frequency of *lacI* and/or *Hprt* mutations in mice, rats, and humans (Segerback, 1990; Walker et al., 1992, 2000; IARC, 1994; Sisk et al., 1997; Tates et al., 1999; Wu et al., 1999; van Sittert et al., 2000; Melnick, 2002). In one study, human EO exposure correlated with elevated *ras* and *p53* expression (Ember et al., 1998). Genetic damage and mutations are thought to play a critical role in the induction of cancer by alkylating agents, such as EO (Miller and Miller, 1966). *N* 7-(2-hydroxyethyl) guanine (HEG) is the major EO-induced adduct formed in rodents and humans (Tates et al., 1991; Li et al., 1992; Walker et al., 1992). The frequent targeting of guanine in the lung and Harderian gland neoplasms in this study suggests that formation of guanine adducts is a mechanism involved in the induction of these neoplasms.

Point mutations in *ras* are most common at codons 12, 13, and 61, which abolish GTPase activity rendering constitutively activated *ras* signalling (Mammas et al., 2005). *Ras* mutations are common in several human neoplasms including lung tumors in which K-*ras* mutations occur in ~30% of all non-small cell lung cancers (Sagawa et al., 1998). K-*ras* mutations also occur in human uterine carcinomas although the incidence tends to be more variable ranging from 0 to 64% (Mammas et al., 2005). As in this study, K-*ras* mutations in human lung and uterine neoplasms most commonly occur in codon 12 (Sagawa et al. 1998; Mammas et al., 2005). Codon 61 K-*ras* mutations are reportedly rare in human uterine carcinomas (Semczuk et al., 2001).

The incidence and spectrum of K-*ras* mutations in spontaneous lung and HG neoplasms from this study were comparable to that previously reported in B6C3F1 mice (Sills et al., 1999; Hayashi et al., 2001). Interestingly, the profile of K-*ras* mutations in the EO-induced neoplasms from this study was different from that described for many other chemically induced lung and HG neoplasms. This suggests that EO induces a chemical-specific signature. Previous studies have identified K-*ras* mutations in lung and HG neoplasms induced by chloroprene-, isoprene- and 1,3-butadiene, however, these mutations were predominantly localized to codon 61 rather than codons 12 and 13 as was seen in this study (Goodrow et al., 1994; Hong et al., 1997; Sills et al., 1999). Additional studies looking at K-*ras* mutations in 1-Amino-2, 4-dibromoanthraquinone- and urethane-induced lung neoplasms in B6C3F1 mice also revealed a predominance of codon 61 mutations (Nuzum et al., 1990; Kawano et al., 1995; Hayashi et al., 2001). The incidence of K-*ras* mutations in mouse uterine neoplasms is poorly characterized, although one study reported that *ras* mutations were rare in N-methyl-N-nitrosurea and 17β-estradiol-induced uterine adenocarcinomas in mice (Murase et al., 1995).

In summary, we found a high incidence of K-*ras* mutations in EO-induced lung, HG and uterine neoplasms in B6C3F1 mice. The prominent targeting of guanine bases in the lung and HG neoplasms suggests that direct DNA damage due to the formation of EO induced *N* -guanine adducts is a likely mechanism of action. In addition, this is to our knowledge, the first study in mice to show an increased incidence of K-*ras* mutations in uterine carcinomas. Overall, our data suggests that EO can specifically target the K-*ras* gene in multiple tissue types and that this event is a critical component of EO-induced tumorigenesis in B6C3F1 mice and has potential relevance to equivalent neoplasms in humans.

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Abbreviations

EO
ethylene oxide

PCR
polymerase chain reaction

DNA
deoxyribonucleic acid

RNA
ribonucleic acid

A, C, G, T
adenine, cytosine, guanine, thymine

NTP
National Toxicology Program

HG
Harderian gland

ddNTP

dideoxynucleotide

HEG

N 7-(2-hydroxyethyl) guanine

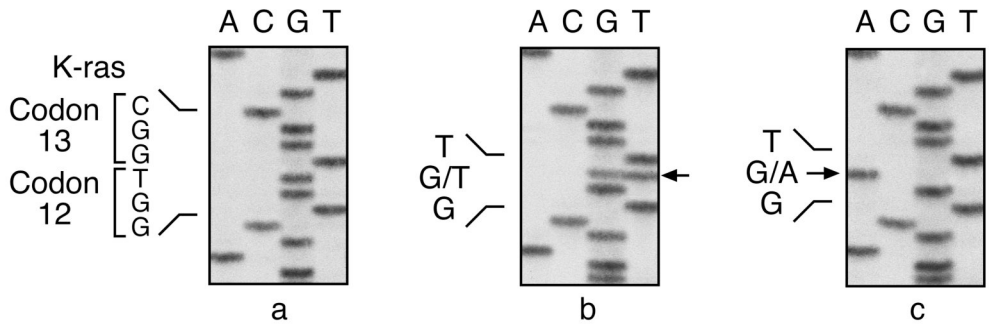

Figure 1.

Identification of K-*ras* mutations at codon 12 in ethylene oxide-induced lung neoplasms from B6C3F1 mice by cycle sequencing analysis. A, C, G, and T represent the 4 nucleotides in DNA, adenine, cytosine, guanine, and thymine, respectively. Sequencing panels are from left to right: (a) normal K-*ras* codon 12 sequence (GGT) from control B6C3F1 lung. (b–c) mutated sequences from lung neoplasms. Arrows point to mutant bands in each sequence. The wild-type allele is invisible in panel (c).

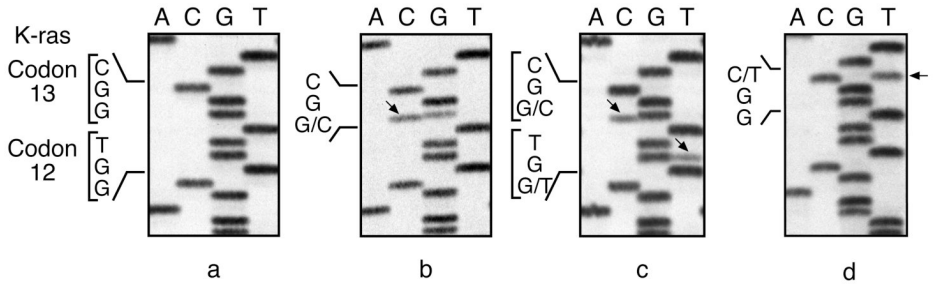

Figure 2.

Identification of K-*ras* codon 12 and 13 mutations in ethylene oxide-induced Harderian gland neoplasms and uterine carcinomas from B6C3F1 mice by cycle sequencing of amplified exon 1. Sequencing panels are from left to right: (a) normal K-*ras* codon 12 sequence (GGT), and codon 13 sequence (GGC). (b–c) mutated sequences from Harderian gland neoplasms, and (d) mutated sequence from uterine carcinoma. Arrows point to mutant bands in each sequence. The wild-type allele is also visible.

Table 1

Incidence of lung, Harderian gland, and uterine neoplasms in ethylene oxide-exposed B6C3F1 mice.[a]

	0 ppm	50 ppm	100 ppm
Lung			
Male			
Alveolar/bronchiolar adenoma	5/50 (10%)	11/50 (22%)	11/50 (22%)
Alveolar/bronchiolar carcinoma	6/50 (12%)	10/50 (20%)	16/50 (32%)
Female			
Alveolar/bronchiolar adenoma	2/49 (4%)	4/48 (8%)	17/49 (35%)
Alveolar/bronchiolar carcinoma	0/49 (0%)	1/48 (2%)	7/49 (14%)
Harderian			
Male			
Papillary cystadenoma	1/43 (2%)	9/44 (20%)	8/42 (19%)
Papillary cystadenocarcinoma	0/43 (0%)	0/44 (0%)	1/42 (2%)
Female			
Papillary cystadenoma	1/46 (2%)	6/46 (13%)	8/47 (17%)
Papillary cystadenocarcinoma	0/46 (0%)	1/46 (2%)	0/47 (0%)
Uterus			
Female			
Uterine carcinoma	0/49 (0%)	1/47 (2%)	5/49 (10%)

[a]Male and female B6C3F1 mice were exposed to 0, 50 or 100 ppm ethylene oxide by inhalation for 6 hours/day, 5 days/week for 2 years.

Table 2

K-*ras* mutations in lung neoplasms from ethylene oxide-exposed B6C3F1 mice.

Treatment	K-ras mutations (%)	Codon 12 (GGT)			Codon 13 (GGC)			Codon 61 (CAA)		
		GAT	TGT	GTT	CGT	AGC	CGC	CGA	CAT	CAC
Control[a]	27/108 (25)	11[b]	5	1	0	0	3	2	4	1
Ethylene oxide[c]	23/23 (100)[d]	2	0	21	0	1	0	1	0	0
A/B adenoma	5/5 (100)	0	0	5	0	0	0	0	0	0
A/B carcinoma	18/18 (100)	2	0	16	0	1	0	1	0	0
50 ppm	8/8 (100)	0	0	8	0	0	0	0	0	0
100 ppm	15/15 (100)	2	0	13	0	1	0	1	0	0

[a] Concurrent controls (8) combined with historical spontaneous lung neoplasms (100) from control B6C3F1 mice.

[b] One mutation was from a concurrent control.

[c] Male and female B6C3F1 mice were exposed to 0, 50, or 100 ppm ethylene oxide by inhalation 6 hours/day, 5 days/week for 2 years.

[d] Two neoplasms had 2 mutations.

Table 3

K-*ras* mutations in Harderian gland neoplasms from ethylene oxide-exposed B6C3F1 mice.

Treatment	K-ras mutations (%)	Codon 12 (GGT)		Codon 13 (GGC)		Codon 61 (CAA)		
		GAT	TGT	CGC	AGC	CTA	CGA	CAG
Control[a]	2/27 (7)	0	0	0	0	2	0	0
Ethylene oxide[b]	18/21 (86)[c]	1	8	15	1	1	3	1
Cystadenoma	18/20 (90)	1	8	15	1	1	3	1
Cystadenocarcinoma	0/1 (0)	0	0	0	0	0	0	0
50 ppm	8/11 (73)	0	5	7	1	1	2	0
100 ppm	10/10 (100)	1	3	8	0	0	1	1

[a] Concurrent controls (2, with no mutations detected) combined with historical spontaneous Harderian gland neoplasms (25) from control B6C3F1 mice.

[b] Male and female B6C3F1 mice were exposed to 0, 50, or 100 ppm ethylene oxide by inhalation 6 hours/day, 5 days/week for 2 years.

[c] Several neoplasms had 2 or 3 mutations.

Table 4

K-*ras* mutations in uterine carcinomas from ethylene oxide-exposed B6C3F1 mice.

Treatment	K-*ras* mutations (%)	Codon 12 (GGT)	Codon 13 (GGC → GGT)	Codon 61 (CAA)
Ethylene oxide[a]				
Uterine carcinoma	5/6 (83)	0	5	ND
50 ppm	1/1 (100)	0	1	ND
100 ppm	4/5 (80)	0	4	ND

[a] Male and female B6C3F1 mice were exposed to 0, 50, or 100 ppm ethylene oxide by inhalation 6 hours/day, 5 days/week for 2 years.

ND—not done.