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A Review of the Molecular Mechanisms of Chemically-Induced Neoplasia in Rat and Mouse Models in National Toxicology Program Bioassays and Their Relevance to Human Cancer

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Abstract

Tumor response in the B6C3F1 mouse, F344 rat, and other animal models following exposure to various compounds provides evidence that people exposed to these or similar compounds may be at risk for developing cancer. Although tumors in rodents and humans are often morphologically similar, underlying mechanisms of tumorigenesis are often unknown and may be different between the species. Therefore, the relevance of an animal tumor response to human health would be better determined if the molecular pathogenesis were understood. The underlying molecular mechanisms leading to carcinogenesis are complex and involve multiple genetic and epigenetic events and other factors. To address the molecular pathogenesis of environmental carcinogens, we examine rodent tumors (e.g., lung, colon, mammary gland, skin, brain, mesothelioma) for alterations in cancer genes and epigenetic events that are associated with human cancer. Our NTP studies have identified several genetic alterations in chemically induced rodent neoplasms that are important in human cancer. Identification of such alterations in rodent models of chemical carcinogenesis caused by exposure to environmental contaminants, occupational chemicals, and other compounds lends further support that they are of potential human health risk. These studies also emphasize the importance of molecular evaluation of chemically induced rodent tumors for providing greater public health significance for NTP evaluated compounds.

Introduction

Each year the National Toxicology Program (NTP) receives nominations of substances deemed of potential human health concern from federal, public, and other sources. The NTP then reviews and selects compounds to be evaluated for potential toxicologic or carcinogenic effects. These compounds range from herbal supplements to occupational chemicals to environmental contaminants. The B6C3F1 mouse, F344 rat, and other mouse and rat models have been used as models to assess effects of these compounds through multiple routes of exposure, including feed, gavage, inhalation, and topical administration, and pathology data gained from subchronic (90 day) and chronic (2 year) NTP studies is used for hazard identification in order to assess the toxicologic and carcinogenic effects of these compounds in rodents.

Using available tissues from NTP studies that exhibit a tumorigenic response, our laboratory evaluates genetic and epigenetic alterations in major classes of oncogenes and tumor suppressor genes in spontaneous and chemically induced rodent neoplasms. The data presented here includes the evaluation of several NTP studies performed between 2004–2008 for which molecular studies were undertaken. Understanding the underlying molecular pathogenesis of cancer in these studies will help determine if the response in the rodent is similar to or different from that of humans. In this way, a major goal of these studies was to relate oncogenic events that occur in the rodent as a result of chemical exposure to changes present in the human disease and make conclusions about potential human cancer risk.

Another goal of these studies was to distinguish chemical-specific tumor responses from spontaneous events, and to determine if signature mutation patterns that occur in rodents following chemical exposure are similar to patterns relevant to cancer in humans.

1. Colon Cancer

Colon cancer is the third leading cause of cancer deaths in the United States. The pathogenesis of colon cancer is a multi-step process involving activation of oncogenes, loss of tumor suppressor gene function, and dysfunction of DNA repair. Loss of function of the APC tumor suppressor gene or β-catenin (*CTNNB1*) gene mutation are early events in the development of colon cancer in humans (Bienz and Clevers 2000; Kinzler and Vogelstein 1996; Peifer 1997). In the WNT signaling pathway, APC forms a large protein complex with β-catenin, axin, and glycogen synthase kinase-3β (GSK-3β) as a result of WNT activation, and ultimately results in β-catenin phosphorylation and proteosomal degradation. With loss of function of APC due to mutation, accumuation of β-catenin occurs, followed by activation of nuclear transcription factors and increases in levels of target proteins such as MYC and cyclin D1. However, mutation of the *CTNNB1* gene without loss of the *APC* gene also results in accumulation of ß-catenin protein and increased WNT signaling in a subset of colon cancers (Davies *et al.* 2005; Fearon and Vogelstein 1990; Fodde *et al.* 2001; Mirabelli-Primdahl *et al.* 1999). The genetic alterations involved in familial adenomatous polyposis (FAP), leading to colorectal cancer, have been well documented and in addition to alterations of the *APC* gene there are mutations in the *KRAS* oncogene, p53 tumor suppressor gene (*TP53*), and *TGFB* growth regulatory genes (Bos *et al.* 1987; Fearon and Vogelstein 1990; Johnstone *et al.* 2002). In addition to known genetic causes, environmental factors may contribute to the estimated 130,000 new cases of colon cancer per year in the US (Sills *et al.* 2004).

o-Nitrotoluene induced-large intestinal cancer—o-Nitrotoluene is used to synthesize agricultural and rubber products, azo and sulfur dyes, and dyes for cotton, wool, silk, leather and paper (Dunnick *et al.* 2003; Huff *et al.* 1985). Low level contamination of rivers and drinking water has been determined through environmental surveys (US EPA, 1976). Given the fact that o-nitrotoluene is a known rodent carcinogen and that low levels of this contaminant exist in the environment, the chemical clearly poses a potential human health risk.

The National Toxicology 2-year carcinogenicity bioassay determined that exposure of B6C3F1 mice to o-nitrotoluene resulted in an increased incidence of cecal carcinomas (NTP 2002). This exposure model provided the first cecal tumor response in mice, and an opportunity to evaluate the morphology and molecular profile of oncogenes, and tumor suppressor genes, that have relevance to the human disease (Sills *et al.* 2004). The induced mouse carcinomas were morphologically and immunohistochemically similar to human colonic adenocarcinoma (Goldstein *et al.* 2000), characterized by clusters and tubules of poorly differentiated, CK7 positive, CK20 negative, tall columnar epithelial cells that invaded into and through the muscle wall. Furthermore, accumulation of β-catenin protein was detected in 80% of the mouse cecal carcinomas, and 73% of the carcinomas showed expression of cyclin D1 and TP53 protein by immunohistochemistry. There was no significant change in the expression of APC protein in the cecal carcinomas.

Mutational analysis revealed multiple mutations of the *Kras*, *Tp53*, and β-catenin (*Catnb*) genes in cecal carcinomas from B6C3F1 mice exposed to o-nitrotoluene. Exon 2 (corresponding with exon 3 in humans) *Catnb* mutations were present in 100% of carcinomas, and mutations in *Tp53* and *Kras* occurred in a majority of tumors (Sills *et al.* 2004). These data showed that genetic alterations in the mouse large intestinal carcinomas

resulted in activation of signal transduction (*Kras* and *Catnb*) and disruption of cell-cycle control (*Tp53*, cyclin D1), hallmarks of human colon cancer. Considering that 100% of cecal carcinomas had mutations in the *Catnb* gene, it is likely that dysregulated expression of β-catenin protein plays a role in the pathogenesis of these mouse cecal carcinomas, as is the case in human colon cancer.

These data correlate with the hypothesis that large intestinal epithelium in the mouse, similar to the case in humans, must acquire multiple mutations for transformation, ultimately culminating in the full malignant phenotype of the adenocarcinoma. The activation of the *Kras* oncogene provides growth-promoting signals, the loss of *Tp53* results in unregulated growth and DNA repair defects, and activation of both *Catnb* and *Kras* results in increased cyclin D1 expression (Hanahan and Weinberg 2000; McCormick 1999). The interaction between these various mutations and their impact on signaling pathways ultimately culminates in the full malignant phenotype of cecal carcinomas.

2. Lung Cancer

Lung cancer is the most commonly diagnosed cancer in the world, and the leading cause of cancer death worldwide (Hong *et al.* 2008). Lung cancer can be divided into non-small-cell lung cancer (80–85%) and small-cell lung cancer (15–20%) based upon histopathologic features (Herbst *et al.* 2008; Wakamatsu *et al.* 2007). Non-small-cell lung cancer can be further divided into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma, of which adenocarcinoma is the most prevalent (Husain *et al.* 2005; Wakamatsu *et al.* 2007). Most lung cancers in humans are non-small-cell lung cancers resulting from genetic and epigenetic damage from chronic exposure to tobacco smoke carcinogens (Hecht 1999; Herbst *et al.* 2008), but the frequency of adenocarcinoma is on the rise in non-smokers (Husain *et al.* 2005). The pathophysiology of pulmonary tumors in general is complex, and relatively little is known about the exact genetic mechanisms underlying the earliest stages of this multi-step process. Mouse models have been utilized for studying carcinogenesis of human lung cancers, and are both histologically similar and share many of the major genetic alterations detected in human pulmonary adenocarcinoma (Wakamatsu *et al.* 2007), including activation of the *KRAS* protooncogene (Meuwissen and Berns 2005; Nikitin *et al.* 2004) and inactivation of the *TP53* tumor suppressor gene (Horio *et al.* 1996; Jackson *et al.* 2005; Takahashi *et al.* 1989). *Kras* mutation is the most common molecular alteration identified in mouse lung adenomas and carcinomas (Ton *et al.* 2007), and occurs in 30–50% of human lung adenocarcinomas. The cumene mouse model of lung carcinogenesis recapitulates many of the molecular alterations found in the human disease.

Cumene-induced lung tumors in B6C3F1 mice—Cumene, or isopropylbenzene, is a component of crude oil used primarily in the production of acetone and phenol (Hong *et al.* 2008). Since the annual production of cumene is increasing in the United States, and there is significant occupational exposure by inhalation, it was nominated for study by the NTP. The 2 year cumene carcinogenicity bioassay demonstrated a significant increase in the incidence of alveolar/bronchiolar adenomas and carcinomas in B6C3F1 mice (Hong *et al.* 2008; NTP 2007). Since certain chemicals often induce specific patterns of activation of *Ras* oncogenes or inactivation of *Tp53* tumor suppressor gene function compared to spontaneous neoplasms in mice (Sills *et al.* 2004; Sills *et al.* 1999b), and these genetic mutations are considered important in the pathogenesis of human lung cancer, we evaluated spontaneous and cumene-induced lung neoplasms for mutations in *Kras* and *Tp53* genes, as well as chemical-specific mutations that could function as biomarkers of chemical exposure with potential human health importance.

Cumene-induced tumors had a significantly higher incidence of *Kras* mutations (87% vs 28%) and *Tp53* mutations (52% vs. 0%) compared to spontaneous lung tumors (Hong *et al.* 2008). While alterations in TP53 protein were not observed in normal lung or spontaneous lung tumors, increased TP53 protein expression was detected by immunohistochemistry in 56% of cumene-induced neoplasms. Additionally, there was loss of heterozygosity (LOH) on chromosome 4 near the *p16* gene and on chromosome 6 near the *Kras* gene in a subset of cumene-induced lung carcinomas, while there was no LOH observed in spontaneous carcinomas or normal lung tissue (Hong *et al.* 2008).

To distinguish gene expression patterns based on the presence or absence of *Kras* and *Tp53* mutation, spontaneous and cumene-induced tumors were analyzed by microarray (Wakamatsu *et al.* 2008). Principal component analysis was able to segregate pulmonary carcinomas induced by cumene into groups with and without *Kras* mutations. Following this, genes associated with the ERK-MAPK signaling pathway were shown to be significantly upregulated in tumors with *Kras* mutations, as well as a number of genes associated with tumor malignancy (invasion and metastasis) such as *Krt8*, *Krt18*, *Lasp1*, *Sdc1*, *Ccnd1*, and *Mmp14*, suggesting that cumene-induced carcinomas with *Kras* mutations have greater malignant potential than those without mutations (Wakamatsu *et al.* 2008). SAFE (significance analysis of function and expression) analysis further showed that carcinomas with *Kras* mutations had changes in expression of histone deacetylases (HDACs), suggesting histone modification as an epigenetic mechanism of carcinogenesis in cumene-induced lung carcinomas (Wakamatsu *et al.* 2008).

Our gene expression analysis suggested the formation of alveolar/bronchiolar carcinomas in cumene-exposed mice typically involves mutation and activation of *Kras*, with activation of the MAPK pathway and dysregulation of a number of genes associated with tumor malignancy. In cumene-induced tumors with and without *Kras* mutation, there was downregulation of a number of tumor suppressor genes (*Ptprd*, *Igsf4a*, *Fhl1*, *Pdzd2*, *Cdkn2d*, *Cdh5*, *Loxl1*, and *Akap12*) and genes associated with inhibition of invasion (*Reck*, *Gsn*, *Lims2*, *Cav1*, and *Gpx3*). In cumene-induced tumors with *Kras* mutations, there was decreased expression of additional genes associated with inhibition of cell motility and cell proliferation (*Igfbp4*, *Sod3*, *Rb1*, *Cebpd*, *Vwf*, and *Dlc1*) and genes associated with patient survival (*Cyr61*and *Enpp2*). Alternatively, there was increased expression of genes known to increase invasion and metastasis (*Krt18*, *Krt8*, *Lasp1*, *Mif*, *Mmp14*, and *Tacstd1*), inhibit apoptosis (*Areg*, *Cks1b*), increase angiogenesis (*Slc2a1*, *Gnb2l1*, and *Ptges*), and increase metastatic potential (*Sdc1* and *Ccnd1*) (Wakamatsu *et al.* 2008). Additionally, cluster analysis of genes generally associated with HDAC regulation revealed a stronger association with tumors with *Kras* mutation.

These results suggest that in cumene-induced pulmonary tumors in mice, DNA damage and genomic instability leading to *Kras* and *Tp53* dysregulation leads to upregulation of pathways associated with the development of lung cancer in cumene-exposed mice, and that tumors resulting from mutations in *Kras* possess a gene expression associated with a greater degree of malignancy. Additionally, the molecular alterations in cumene-induced lung tumors appear to affect similar pathways as those in the human disease, suggesting that the tumor response in mice may be relevant to human lung cancer.

AZT-induced lung tumors in CD-1 mice—3-azido-3-deoxythymidine (AZT) was the first drug approved by the US Food and Drug Administration (FDA) for treatment of HIV-1 in adults and children (Bhana *et al.* 2002; Vivet-Boudou *et al.* 2006). AZT also reduces vertical transmission of HIV-1 during pregnancy by 70% (Connor *et al.* 1994). However, there are concerns about its carcinogenic potential, given that it induces gene mutations and

chromosomal damage both in vitro and in laboratory animals via direct or in utero exposure (Olivero *et al.* 1997; Phillips *et al.* 1991).

Following exposure in utero to high doses of AZT, male Swiss (CD-1) mouse offspring had a significantly increased incidence of alveolar/bronchiolar adenomas and carcinomas (NTP 2004a). Since *Kras* and *Tp53* mutation are important in the pathogenesis of human lung cancer, lung tumors from male CD-1 mice were evaluated for *Kras* and *Tp53* point mutations by DNA sequencing of formalin-fixed, paraffin-embedded tumors. The results of mutational analysis revealed that a majority of the AZT-induced tumors contained mutations in *Kras* (66%) and *Tp53* (84%) (Hong *et al.* 2007a). The primary *Kras* mutation was a G➔T transversion at codon 12, and the predominant *Tp53* mutations were A➔T transversions in exon 8, codon 285 and T➔A transversions in exon 6, codon 198. Although the incidence of AZT-induced lung tumors in females was not statistically significantly increased above controls, a majority of these tumors contained the same *Kras* mutation (61%) as found in male lung tumors. *Kras* and *Tp53* mutations were not present in normal lung or spontaneous alveolar/bronchiolar lung tumors from CD-1 mice (Hong *et al.* 2007a).

Our laboratory determined that AZT-induced pulmonary tumors in both male and female CD-1 mice resulted from molecular alterations in the same pathways as human lung cancer, and that genotoxic damage from AZT or its metabolites may contribute to the development of lung tumors in these mice (Hong *et al.* 2007a; Koujitani *et al.* 2008). This suggests that the response in the mouse may be of relevance to the mechanism of carcinogenicity in humans. In addition, these data are in agreement with other transplacental carcinogenicity studies (Olivero *et al.* 2001), in vitro models (Meng *et al.* 2002; Meng *et al.* 2000) and rodent (Diwan *et al.* 1999; Poirier *et al.* 2003; Von Tungeln *et al.* 2002) and human (Olivero *et al.* 1999) fetuses exposed to AZT. Taken together, these data suggest that human infants exposed to AZT in utero may have an increased risk of lung cancer later in life.

Ethylene oxide-induced lung and other tumors in B6C3F1 mice—Ethylene oxide (EO) is commonly used as a sterilization agent in the health care industry, and it is also used to produce several chemicals. As a result of occupational exposure in the health care industry, it is estimated that 75,000 workers are at risk of exposure (IARC 1994; NTP 2004b; Recio *et al.* 2004). It has carcinogenic activity in rodents where it induces tumors in the lung, harderian gland, and uterus, and causes DNA damage in human cells (IARC 1994; NTP 1987; Nygren *et al.* 1994). Chronic human exposures have been suggested to be associated with leukemias, lymphomas, breast cancer, and stomach cancer (Norman *et al.* 1995; Shore *et al.* 1993; Stayner *et al.* 1993; Steenland *et al.* 1991; Teta *et al.* 1993).

To evaluate the potential human risk from EO, our laboratory examined EO-induced tumors in the lung, harderian gland, and uterus from exposed mice for *Kras* mutations. While this mutation was found in 25% (27/108) of spontaneous lung tumors, 100% (23/23) of EO-induced lung tumors had *Kras* mutations (Hong *et al.* 2007b). The most common mutations were codon 12 G➔T transversions, which were infrequent in spontaneous tumors. *Kras* mutations were also very common in EO-induced harderian gland tumors (18/21, 86%), and infrequent in spontaneous tumors (2/27, 7%). In EO-induced tumors, codon 13 G➔C and codon 12 G➔T transversions were common, but spontaneous tumors did not possess these mutations. Likewise, in uterine tumors, *Kras* mutations occurred in a majority (5/6, 83%) of EO-induced uterine carcinomas, and all were codon 13 C➔T transitions.

These data shows that mutations in *Kras* are very common and an important step in the pathogenesis of EO-induced lung, harderian gland, and uterine tumors in the B6C3F1 mouse (Hong *et al.* 2007b). Furthermore, since EO-induced tumors in the mouse share genetic

alterations with the human disease, exposure to this chemical may pose a significant human health risk, and further evaluation of its effects in humans is warranted.

1,3-butadiene or chloroprene-induced lung and brain tumors from B6C3F1 mice—1,3-Butadiene and chloroprene are used in the production of synthetic rubber (Himmelstein *et al.* 1997; NTP 1993, 1998). Additionally, 1,3-butadiene is a combustion product of fossil fuel and wood, and is present in automobile exhaust and tobacco smoke. Both chemicals are multi-organ carcinogens in rodents, most often causing lung tumors in female mice (Huff *et al.* 1985; Melnick *et al.* 1999). Other tumors induced by these chemicals include lymphoma, hemangiosarcoma, and harderian gland tumors. Epidemiological studies in humans have correlated occupational exposure to 1,3-butadiene with increased leukemia and lymphoma incidence (Macaluso *et al.* 1996; Santos-Burgoa *et al.* 1992). Based upon these and other in vitro and in vivo findings, the NTP Report on Carcinogens (ROC) has listed 1,3-butadiene as a “known human carcinogen,” and chloropene as “reasonably anticipated to be a human carcinogen” (NTP 2000).

Mutational analysis indicated that a large proportion of lung tumors induced by these chemicals harbored *Kras* mutations (Ton *et al.* 2007). A majority (34/51, 67%) of lung tumors induced by 1,3-butadiene had codon 13 G➔C transversions. Likewise, a large proportion (19/25, 76%) of lung tumors arising from chloroprene exposure had *Kras* mutations, except that mutation profile was different, with half of the tumors harboring codon 61 A➔T mutations. These mutations lead to the formation of guanine and adenine DNA adducts in the lung, and may play critical roles in the development of lung tumors induced by these chemicals. In addition to *Kras* mutations, there was a high frequency of loss of heterozygosity (LOH) in these tumors in the region of *Kras* on chromosome 6, suggesting loss of the wild-type allele. The findings of *Kras* mutation and wild type allele loss in these mouse lung tumors are similar to molecular alterations in some human lung adenocarcinomas, and suggest that wildtype *Kras* may act as a tumor suppressor gene (Zhang *et al.* 2001). Further inhalation studies of 1,3-butadiene and chloroprene may provide a model system for understanding certain types of lung cancer in humans.

Along with the increased incidence of lung tumors, harderian gland tumors, hemangiosarcomas, and lymphomas, exposure to 1,3-butadiene surprisingly resulted in an increased incidence of brain tumors in B6C3F1 mice (Kim *et al.* 2005a). While the central nervous system is a rare target for carcinogenesis in NTP bioassays (Ton *et al.* 2007), there was an increase in glioma and neuroblastoma formation with locational and morphologic similarities to their human counterparts (Kleihues *et al.* 2002). Many of the molecular alterations in brain tumors resulting from 1,3-butadiene exposure in mice are similar to that seen in human brain tumors. First, alterations in the *Tp53* tumor suppressor gene were common. *TP53* mutation is common in human glial tumors, dysfunction of which has been suggested to be an early event in glial tumorigenesis in humans (Holland 2001). There were mis-sense mutations in half (3/6) of malignant gliomas, and in both (2/2) neuroblastomas examined. These tumors were always associated with loss of heterozygosity of the *Tp53* gene. Most of the *Tp53* mutations found in this study were G➔A transitions, similar to those seen in human glioblastoma, in which up to 67% of tumors have *Tp53* mutations, with the majority being G➔A transitions (Fulci *et al.* 2000; Watanabe *et al.* 1996). Loss of the *P16 (CDKN2A)* tumor suppressor gene has been observed in human astrocyte cancer cell lines in vitro (Bachoo *et al.* 2002) and similarly, LOH was noted at the *Ink4a/Arf* tumor suppressor locus in all mouse gliomas and neuroblastomas, indicative of loss of the *Cdkn2a* tumor suppressor gene. These data show that the molecular changes induced by exposure to 1,3-butadiene are similar to those present in the human disease, and suggest that environmental exposure to this chemical might pose a significant cancer risk. This is the first report to our knowledge in which multiple mutations of tumor-associated genes observed in

human brain tumors have been detected in mouse brain tumors following exposure to an environmental chemical.

3. Skin Cancer

Squamous cell carcinoma (SCC) is the second most common skin cancer in humans, accounting for 20% of all malignancies of the skin (McGuire *et al.* 2009; Wade and Ackerman 1978). Ultraviolet light plays a significant role in the development of SCC, and both UVA and UVB may contribute to skin cancer; both UVA and UVB cause DNA damage, but UVB also causes injury to Langerhans cells, resulting in compromised local immune surveillance (de Gruijl 2000; McGuire *et al.* 2009). Other common causes of SCC include thermal injury, human papillomavirus, chronic irradiation dermatitis, and chemical carcinogenesis (McGuire *et al.* 2009).

Squamous Cell Carcinomas in HRA/Skh Mice induced by 8-Methoxypsoralen (8-MOP) and UVA Radiation—Treatment with 8-methoxypsoralen (8-MOP) and ultraviolet radiation (primarily UVA), called PUVA therapy, has been used for decades to treat various skin diseases such as psoriasis, vitiligo, and cutaneous T-cell lymphoma. The mechanism of action is through photosensitization by psoralen, which covalently binds pyrimidine bases through a photocycloaddition reaction following UVA exposure (Gasparro *et al.* 1997). However, epidemiological evidence has shown a significant increase in skin tumors, predominantly squamous cell carcinoma (SCC), in chronically treated individuals. A consistent mutation pattern of *TP53* has been shown in vivo and in vitro as a result of the direct action of UV light on DNA (Inga *et al.* 1998; Monti *et al.* 2000; Nataraj *et al.* 1997; Santamaria *et al.* 2002), rather than photoactivation of the psoraslen treatment, showing a direct involvement of the *TP53* gene in human skin tumorigenesis. The *Hras* proto-oncogene is also known to play a role in chemically induced skin carcinogenesis in rodents (Mangues and Pellicer 1992), and is a known mutational target in PUVA induced SCC in humans (de Gruijl *et al.* 2001; Sills *et al.* 1999a).

The NTP performed a study in mice exposed to PUVA therapy to test its carcinogenic potential (Dunnick *et al.* 1991). The results showed that PUVA therapy caused a significant increase in SCC of the skin in the hairless HRA/Skh mouse. Our laboratory then examined the *Tp53* and *Hras* mutational pattern as well as Tp53 and PCNA protein expression in hyperplastic and neoplastic squamous cell lesions from the NTP study (Lambertini *et al.* 2005). By immunohistochemistry, Tp53 and PCNA protein was detected in 3/16 (19%) of hyperplastic lesions and 14/17 (82%) of SCCs in animals that were treated with both 8-MOP and UVA (Lambertini *et al.* 2005). In UVA and 8-MOP treated animals with SCC, 15/17 (88%) had mutation of *Tp53*, and 93% of those animals had mutation in exon 6 (93%). However, *Tp53* mutations in SCCs from human patients treated with PUVA predominantly occur in exons 5, 7, and 8. Additionally, there was no evidence of *Hras* mutations in either hyperplastic skin lesions or SCCs. Overexpression of Tp53 and PCNA protein was not observed in normal mouse skin.

The study described above showed that photoactivated 8-MOP induced an increased incidence of SCCs with a high frequency of *Tp53* mutations in HRA/Skh mice that were dosed orally, and given similar UV intensity as would occur in human patients (Dunnick *et al.* 1991; Lambertini *et al.* 2005; Nataraj *et al.* 1997). The mutagenic effect of PUVA on the *Tp53* tumor suppressor gene may lead to a conformational modification and inactivation of the Tp53 protein, which is considered a critical step in PUVA-induced skin carcinogenesis. Although the *Tp53* mutational frequency and patterns were different from those reported in PUVA-type tumors, the carcinogenic risk of PUVA treatment should not be underestimated,

and preventative measures should be taken when this clinical approach is used (Lambertini *et al.* 2005).

4. Malignant Mesothelioma

In humans, mesothelioma is a malignant proliferation of the lining of body cavities, most commonly the pleura, but also occurs in the peritoneum and pericardium, as well as the tunica vaginalis (Musti *et al.* 2006; Spugnini *et al.* 2006). Human malignant mesothelioma is most commonly caused as a result of exposure to asbestos (Carbone and Bedrossian 2006; Suzuki and Yuen 2002). However, whether asbestos induces mesothelioma by direct interaction with pleural cells, or indirectly through the generation of toxins and reactive oxygen species that may result in genotoxicity or dysregulation of other cellular pathways is not clear.

The ability for asbestos to stimulate mesothelial proliferation through induction of AP-1 and activation of NF-κB through TNFα released by macrophages in response to inflammation has been established (Carbone and Bedrossian 2006). However, development of human malignant mesothelioma is mediated by several additional genetic defects that result in loss or down-regulation of tumor suppressors or overexpression of oncogenes. Loss of cyclin dependent kinase function (*CDKN2A, CDKN2B*), leading to Tp53 and pRB inhibition is well characterized in human malignant mesothelioma, as is overexpression of genes associated with cellular growth and survival (*IGF, IGFR, EGFR, FOS, JUN*), anti-apoptotic pathways (*BCL2*), angiogenesis (*VEGF, COX2*), loss of tumor suppressor genes either through mutation (*NF2, WT1*), or hypermethylation (*APC, CDNK2A, CDNK2B, RASSF1A*) (Christensen *et al.* 2008; Kumar and Kratzke 2005; Spugnini *et al.* 2006; Whitson and Kratzke 2006).

Gene Expression in O-nitrotoluene and Bromochloracetic Acid-Induced Peritoneal Mesothelioma in F344 Rats—This study was performed to identify and characterize major carcinogenic pathways involved in rat peritoneal mesothelioma (RPM) formation following treatment with o-nitrotoluene (o-NT) or bromochloracetic acid (BCA) in F344 rats (Kim *et al.* 2006). In the F344 rat, spontaneous mesothelioma occurs at an incidence of 2.7–3.6%, and primarily affects the peritoneal cavity, and more rarely the thoracic cavity (Haseman *et al.* 1990; Kim *et al.* 2006). Many chemicals in NTP studies have been shown to increase the incidence of mesothelioma in F344 rats, including o-nitrotoluene (o-NT) and bromochloroacetic acid (BCA) (NTP 2002). Tumors are induced by o-NT in multiple sites, and our laboratory has previously shown that o-NT-induced cecal carcinomas have alterations in the WNT/Beta-catenin signaling pathway, KRAS/MAP kinase pathway, Tp53 pathway, and cyclin D1 (Sills *et al.* 2004).

This study was conducted to determine the major carcinogenic pathways at play in the development of RPMs due to o-NT or BCA exposure in F344 rats. Over 20,000 genes were evaluated in chemically induced rat peritoneal mesotheliomas using oligo arrays, and comparing the data to a non-transformed rat mesothelioma cell line. Analysis revealed dysregulation of 169 cancer-related genes, involving numerous biological processes such as cell cycle progression, growth and proliferation, apoptosis, invasion, and metastasis. Importantly, there are many pathways important in the development of mesothelioma in humans that were subsequently identified in chemically induced rat mesotheliomas, including insulin-like growth factor 1 (*Igf1*), p38 MAPK (*Mapk14*), WNT/Beta-catenin, and integrin signaling pathways (Kim *et al.* 2006). This demonstrates that RPMs induced by o-NT and BCA are similar to the human disease at the cellular and molecular level, providing an opportunity to identify genetic pathways that may be of importance in the study of the human disease.

5. Liver Cancer

Hepatocellular carcinoma (HCC) accounts for over 90% of primary hepatic neoplasia (Kim *et al.* 2005b). The pathogenesis of this disease in humans is multifactorial, associated with various infectious agents, carcinogens, environmental and lifestyle factors (Coleman 2003). In B6C3F1 mice, hepatocellular adenoma (HCA) is the most common spontaneous liver neoplasm second to HCC, and occurs more commonly in males than in females (Kim *et al.* 2005b). Hepatoblastoma (HB) is a malignant embryonal tumor affecting predominantly children under three years of age (Koch *et al.* 1999). It is the most common malignant hepatic tumor in children, accounting for 1.5 cases per one million, occurring most often sporadically, but also associated with familial syndromes such as familial adenomatous polyposis coli (FAP) or Beckwith-Wiedemann syndrome (Ishak and Glunz 1967; Kim *et al.* 2005b; Steenman *et al.* 2000). Hepatoblastomas in mice rarely occur spontaneously, while higher incidences may occur in chemically-induced models, and may occur within a pre-existing HCC or HCA (Turusov *et al.* 2002). Various genetic alterations have been reported in the development of liver tumors in humans, including alterations in genes involved in growth and proliferation, oncogenes (Coleman 2003), DNA damage response and cell cycle control genes (Hainaut *et al.* 1998), genes associated with cell-cell interaction and signal transduction (Nhieu *et al.* 1999; Yamamoto *et al.* 2003), as well as epigenetic mechanisms (Cherian *et al.* 2003; Lim 2002; Wong *et al.* 2003). Many of the same genes involved in human hepatocarcinogenesis are implicated in the development of altered foci, hepatocellular adenoma (HCA) and carcinoma, and hepatoblastoma (HB) in mice, emphasizing the importance of the mouse in the study of the human disease (Kim *et al.* 2005b). For example, preneoplastic proliferative liver lesions in humans have increased expression of several growth factors and receptors (*IGFII, HGF, TGFA*) (Coleman 2003; Kiss *et al.* 1997; Lund *et al.* 2004) that have been shown to cause liver tumors in transgenic mouse models (Fausto 1999; Kim *et al.* 2005b). Furthermore, liver tumors may be associated with mutations in oncogenes such as *Hras* in spontaneous or chemically-induced mouse models (Kim *et al.* 2005b), and increased expression of *HRAS, NRAS*, and *KRAS* have been associated with liver tumors and preneoplastic lesions in humans, with associated upregulation of downstream MAP kinase pathway (Coleman 2003). Alterations in the WNT signaling pathway are common in chemically-induced liver tumors, including mutations in β-catenin (*Catnb*), resulting in translocation of β-catenin to the nucleus and cyclin D expression (Anna *et al.* 2003; Kim *et al.* 2005b). Mutations in the β-catenin gene are frequent and early in the pathogenesis of chemically-induced hepatic tumors in mice, and may be chemical-specific in nature (Devereux *et al.* 1999). In the NTP two year bioassay of methyleugenol-induced and oxazepam-induced liver tumors in B6C3F1 mice, there was a 69% and 41% mutation incidence in β-catenin, respectively (Anna *et al.* 2003; von Schweinitz *et al.* 1996). Similarly, following exposure to diethanolamine for two years, B6C3F1 mice developed HCC associated with genetic alterations in the *Catnb* gene; 32% (11/34) adenomas and carcinomas had mutations in exon 2 of the β-catenin gene (Hayashi *et al.* 2003). Similar mutations in exon 3 of the human β-catenin gene have been frequently reported in human HCA (Nhieu *et al.* 1999; Yamamoto *et al.* 2003). Like HCA, hepatoblastomas in mice and humans are also associated with high incidences of β-catenin gene mutation (Anna *et al.* 2000; Jeng *et al.* 2000; Koch *et al.* 1999), including downstream activation of WNT signaling (Anna *et al.* 2003; Takayasu *et al.* 2001). In B6C3F1 mice, 100% (5/5) of hepatoblastomas and 34% (12/35) of HCA had elevated expression of cyclin D1, including 67% (10/15) HCA with β-catenin gene mutation (Anna *et al.* 2003). Other mediators associated with or involved in the regulation of β-catenin (*EGFR, MET, CDH1*) are altered in both human and mouse hepatoblastoma (Anna *et al.* 2003; von Schweinitz *et al.* 1996), illustrating an overreaching role of WNT signaling on hepatoblastoma development.

6. Breast Cancer

According to the American Cancer Society, breast cancer is the most common cancer in women in the United States, and is the second leading cause of cancer mortality (Jemal *et al.* 2008). Several genetic and epigenetic alterations have been implicated in the genesis of breast cancer, and in general breast cancer is divided into hereditary and sporadic forms. Hereditary forms account for approximately 5–10% of breast cancers, and are caused predominantly by mutation in the high-penetrance breast cancer susceptibility genes *BRCA1* and *BRCA2* (Tan *et al.* 2008). Sporadic or non-hereditary cases of breast cancer are associated with a variety of genetic and epigenetic abnormalities that result in dysregulation of growth pathways (*IGF, EGFR, ESR1*), cell cycle regulators (*CDKN1A, CDKN1B, CDKN2A, CCND1*), oncogenes (*ERBB2, MYC*), tumor suppressor genes (*TP53, RB, ATM, CDH1*), and chromatin modifiers (*BMI1*) (Datta *et al.* 2007; Kenemans *et al.* 2004; Lerebours and Lidereau 2002). While *RAS* is mutated in less than 10% of breast cancers (Lerebours and Lidereau 2002), the RAS/RAF/MEK/ERK growth signaling pathway is reported to be frequently activated in breast cancer (McCubrey *et al.* 2007).

P53 and H-ras Mutations in Benzene- and Ethylene Oxide-induced Mammary Carcinoma in B6C3F1 Mice—Benzene and ethylene oxide have been shown to cause cancer at multiple sites in rodents, and have been classified by the National Toxicology Program as human carcinogens. Both chemicals have been shown to cause increased incidence of mammary carcinomas in mouse carcinogenicity studies (IARC 1994; Maltoni and Scarnato 1979; NTP 2004b; Snellings *et al.* 1984). *TP53* and *RAS* are the most frequently mutated genes in human cancers, and based on a high frequency of *TP53* mutation and RAS signaling in human breast cancers, this study was performed to determine the role of *Tp53* and *Hras* in spontaneous, benzene-, and ethylene oxide-induced mouse mammary carcinomas in B6C3F1 mice.

TP53 protein expression was detected in a significant proportion of spontaneous (42%), benzene-induced (6/14, 43%), and ethylene oxide-induced (8/12, 67%) mammary carcinomas by immunohistochemistry (Houle *et al.* 2006). The amount of Tp53 protein detected by semiquantitative analysis was five- to six-fold higher in chemically induced carcinomas compared to spontaneous tumors. DNA mutation analysis detected *Tp53* mutation in a significant number of spontaneous (7/12, 58%), benzene-induced (8/14, 57%), and ethylene oxide-induced carcinomas (8/12, 67%). Twenty-six percent (5/19) of spontaneous, 50% (7/14) of benzene-, and 33% (4/12) of ethylene oxide-induced carcinomas had mutations in *Hras*, and importantly, concurrent *Tp53* mutation was observed when *Hras* mutations were present. Concurrent *Tp53* gene mutations were identified in 71% (5/7) of benzene-induced, 75% (3/4) of ethylene oxide-induced, and 40% (2/5) of spontaneous carcinomas (Houle *et al.* 2006).

The mutation pattern between chemically induced and spontaneous tumors was significantly different. In chemically induced tumors, *Hras* mutations most commonly involved the second base of codon 61, and in 10/11 tumors resulted in an amino acid change from glutamine to leucine or arginine. Mutations in spontaneous tumors however, involved the first base and most often resulted in amino acid changes of glutamine to lysine (Houle *et al.* 2006).

Our results show that although mutations of the *Tp53* and *Hras* genes are relatively common in spontaneous, as well as in chemically induced, mouse mammary carcinomas, the incidence of concurrent *Tp53* and *Hras* mutation is increased in chemically induced tumors (Houle *et al.* 2006). Furthermore, the pattern of mutations in *Tp53* and *HRas* differed between chemically induced and spontaneous mammary carcinomas, suggesting that different mechanisms are involved between these tumors in B6C3F1 mice.

Immunohistochemical Detection of Molecular Alterations

Immunohistochemistry may be used to detect the effects of gene mutation on protein expression and/or localization in chemically induced tumors. Abnormal protein expression, such as overexpression, loss, or abnormal localization may provide evidence of underlying genetic or epigenetic mechanism of tumorigenesis.

Abnormal Localization

As stated previously, the majority of hepatoblastomas in mice have mutations in the β-catenin gene (Anna *et al.* 2000; Kim *et al.* 2005b). This mutation affects the binding region of GSK3B, resulting in prevention of phosphorylation of β-catenin and resultant accumulation within the cytoplasm. Cytoplasmic β-catenin that is not targeted for degradation by the proteasome may translocate to the nucleus where it may associate with LEF1/TCF transcription factors and drive transcription of target genes such as *Ccnd1* and *Myc*. Therefore, evidence of this mutation may be seen with immunohistochemistry as both loss of membrane associated β-catenin protein expression as well as abnormal localization to the nucleus (Hayashi *et al.* 2003). Since nuclear localization may not be present in all tumors with *Catnb* mutation in mice (Devereux *et al.* 1999), mutation analysis including PCR and sequencing should be used to follow up on the identification of the specific mutation.

Overexpression

Gene mutation is often associated with overexpression of a protein product. Several proto-oncogenes are converted to oncogenes through mutation, and induce uncontrolled proliferation through various growth pathways. The *Ras* proto-oncogenes undergo mutation in several different types of cancer, resulting in downstream activation of numerous kinases and anti-apoptosis mediators controlling the proliferation of cells. Using immunohistochemistry, the effects of *Ras* mutation may be detected, such as overexpression of downstream mediators such as MAP kinases and transcription factors that lead to promotion of the cell cycle and cell proliferation (Sills *et al.* 1999a). Western blot analysis should be used to quantify the degree of overexpression, and PCR and gene sequencing targeting the gene hot spots of *H-, N-*, or *Kras* may be performed to identify the specific genetic lesion.

Loss of expression

Loss of expression of a protein product in tumors is often associated with inactivation or deletion mutations in tumor suppressor genes or genes regulating apoptosis. The loss of tumor suppressor function results in loss of cell cycle control and unregulated proliferation. Important tumor suppressors often lost in tumorigenesis are T*p53* and retinoblastoma (*Rb*). *TP53* is the most commonly mutated gene in human cancer. As the “guardian of the genome”, TP53 is responsible for cell cycle control and arrest at the G1 checkpoint to allow time for DNA repair. Mutation of *Tp53* often results in a loss of function of one or both alleles, resulting in a dysregulation of this checkpoint arrest, and continuation through the cell cycle without DNA repair. This allows further mutations to accumulate and results in oncogenic transformation as a result of defective DNA repair. The expression of wildtype TP53 protein in tissues is transient, so it is undetectable by immunohistochemical methods; however, when mutated, a defective, nonfunctional TP53 protein product, which has a long half-life, may accumulate within the nucleus (Gerbes and Caselmann 1993). Therefore, loss of function of the wildtype TP53 protein and overexpression of the mutated form is indicative of genetic mutation. Mutation of other tumor suppressor genes, such as *Cdkn2a* or *Rb1*, may result in decreased or complete absence of protein expression by immunohistochemistry. *Rb1* is an important tumor suppressor gene regulating the cell cycle,

and mutation or loss causes unchecked cell proliferation. CDKN2A inhibits phosphorylation of RB1, thereby preventing cell cycle progression. Mutation in either of these tumor suppressor genes results in decreased or loss of expression of their protein products in cancer cells. PCR analysis to detect loss of one or both alleles or gene sequencing should be used to identify the specific mutation responsible.

Significance of genetic alterations in tumor initiation and progression in rodents

As models of environmental and occupational exposure to carcinogens, investigation of the underlying mechanisms of tumorigenesis in rodents can yield valuable information as to the molecular events taking place in the progression of lesions from preneoplastic lesions to neoplasia. Although metastasis is difficult to study in chemically induced models of neoplasia since it is a relatively infrequent and time-dependent occurrence, it has been extensively studied in genetically engineered mouse models. Knowledge of the genetic lesions occurring from one stage to the next can provide important information regarding the potential pathogenesis of these lesions in humans that are potentially exposed to such compounds. The genetic mutations that occur in rodent models often follow a distinct progression in the development from benign tumors to malignant cancers. Chemically induced and transgenic rodent models are frequently used to model preneoplastic and neoplastic lesions that result from genetic alterations in humans, and such models give insight into mechanisms and potential treatment or provide information on current applications of diagnostic modalities for use in human patients. Many of the genetic alterations we have identified in chemically induced rodent models of cancer mimic changes present in human cancer in terms of stages of initiation and progression from premalignancy to neoplasia.

Initiation

Several of our chemically induced mouse models of lung cancer have alterations in the *Kras* and *Tp53* genes, two genes commonly mutated in human lung cancer. Mutations in *Kras* are believed to be an early initiating event in lung carcinogenesis in humans (Spivack *et al.* 1997; Westra *et al.* 1993b), and mice (Donnelly *et al.* 1996; Horio *et al.* 1996; Spivack *et al.* 1997; Wakamatsu *et al.* 2007). Similarly, in chemically induced rodent liver tumor models as well as human liver tumors, *Ras* mutation is associated with early events in tumorigenesis. For example, increased levels of *HRAS, NRAS*, and *KRAS* have been reported in some pre-neoplastic liver lesions in humans (Coleman 2003), and activated *Kras* and *Hras* oncogenes have been detected in chemically induced mouse liver tumors (Maronpot *et al.* 1995; Reynolds *et al.* 1988). Beta-catenin gene mutation is another important event in mouse and human hepatic tumors that has been shown to be an early initiating event in chemically-induced mouse (Devereux *et al.* 1999) and rat (Yamada *et al.* 1999) hepatocellular carcinogenesis. Expression of insulin growth factor II (IGFII) and transforming growth factor alpha (TGFA) are associated with preneoplastic lesions in humans (Thorgeirsson and Grisham 2002), and contribute to the formation of liver tumors in mouse models, including IGFII expression during early stages of liver carcinogenesis (Lahm *et al.* 2002). Although dysregulation of β-catenin and WNT signaling most often follows mutation of the *APC* gene as an initiating event in human colon cancer, mutation of the β-catenin gene as a primary initiating event also occurs in a subset of colon cancers in humans (Fodde *et al.* 2001; Mirabelli-Primdahl *et al.* 1999), as well as a chemically-induced mouse model of large intestinal (cecal) carcinoma (Sills *et al.* 2004). In addition, *Kras* activating mutations have been shown to be an early initiating event in a rat model of chemically induced colon carcinogenesis (Jacoby *et al.* 1991; Rosenberg *et al.* 2009).

Progression

Mutations in *TP53* are frequently observed in human lung adenocarcinomas (Hwang *et al.* 2003; Westra *et al.* 1993a), as well as mouse lung tumors (Wakamatsu *et al.* 2007), and can act synergistically with *Kras* mutation in the development of certain cancers in mice and humans (Fisher *et al.* 2001; Halevy *et al.* 1990; Pierceall *et al.* 1991; Wakamatsu *et al.* 2007; Wang *et al.* 2006). Mutation of *TP53* occurs late in the course of disease and plays an important role in progression to malignancy in mice (Horio *et al.* 1996; Jackson *et al.* 2005; Wakamatsu *et al.* 2007), and is associated with a poor prognosis in human patients with non-small cell lung cancer (Huang *et al.* 1998). In the liver, *TP53* mutation is associated with later stages in aflatoxin induced hepatocellular carcinogenesis (Liu *et al.* 1996) and advanced stages of tumorigenesis in humans (Hsu *et al.* 1993).

Several other studies have investigated the role of distinct genomic aberrations in the progression from premalignant to neoplastic lesions in chemically induced rodent models of human liver (Longato *et al.* 2009; Tward *et al.* 2007), lung (Hutt *et al.* 2005; Malkinson 1992), colon (Yamada and Mori 2007), mammary gland (Medina 2008; Russo and Russo 1996), skin (Balmain and Pragnell 1983; Brown *et al.* 1990) and other cancers, and the impact of genetically engineered mouse models has continued to increase the understanding of genetic alterations and their effect on carcinogenesis. Thus the occurrence of these mutations in the mouse suggests that chemically induced mouse models of carcinogenesis can in many ways model human tumorigenesis.

Impact on Risk Assessment for Human Health

Several methods are used to identify environmental or occupational hazards that pose significant carcinogenic risks to humans, including in vitro mutagenesis assays and in vivo long-term carcinogenicity assays in rodents, with classification of a compound as a hazard based predominantly on the latter (Reynolds *et al.* 1988). Incidence of neoplasia in a rodent species as a result of chemical exposure provides information on risk assessment for human health; however, it is difficult to determine whether or not an exposure in humans will elicit a similar response, since hazard identification and risk assessment requires the presumption of similarity between rodents and humans (Holsapple *et al.* 2006). This is challenging due to several reasons. One, the metabolic activity of mice and rats may differ significantly not only between one another but also from that of other species such as man. Secondly, there are significant differences in tumor susceptibility between strains of mice as well as rats. Finally, doses administered to rats and mice are often a greater magnitude than that of what humans are exposed to. Therefore, often it is difficult to extrapolate the findings in rodents exposed to chemical carcinogens to that of what may be predicted in humans. What does this mean for human risk assessment? The induction of a tumor response in rodents as a result of exposure to a compound alone is becoming insufficient as a means of prediction of human disease. While this tells us a certain compound is a rodent carcinogen, it does not definitively confirm that the same effect will be seen in humans at the same site or by a similar mechanism. The development of molecular investigation into the underlying genetic and epigenetic mechanisms of carcinogenesis is providing a more detailed and definitive explanation of why cancer occurs in these models. As a result, scientists and regulators may compare genetic and epigenetic mechanisms in rodent models to those known to occur in human cancers in order to develop a more predictive model of chemically induced carcinogenesis in humans. These approaches will continue to enable regulators to make more definitive and confident decisions regarding human exposure to certain compounds, and may remove some of the uncertainty related to species and dose extrapolation of human health risk from rodent carcinogenicity data. Furthermore, the investigation into the molecular pathogenesis of these models should become a mainstay in decision-making

regarding the prediction of human health risks and regulation of various occupational and environmental compounds.

Conclusions and Summary

The use of the mouse and rat as models of chemically induced carcinogenesis, both environmental and occupational, has provided evidence that human exposure to these chemicals may pose a significant health risk for developing cancer. These animal models provide us with tools to decipher the molecular mechanisms that may be at play in the genesis of human cancer, and therefore may be extremely valuable to the understanding of the underlying genetic and non-genetic causes of human cancer. Our group has investigated numerous compounds that pose a potential human cancer risk. From these studies, we have identified several genetic events, and in future studies plan to examine epigenetic pathways responsible for the development of different types of chemically induced tumors in the mouse and rat. Using mutation analysis, gene expression studies, immunohistochemistry, and other allied research techniques, we have identified several factors in the rodent models that are responsible for the generation of cancer. This research has been important in furthering our understanding of the mechanisms of chemically-induced carcinogenesis and the potential risks that certain environmental, occupational, and food-related chemical compounds may have on human health.

Our studies of genetic and epigenetic mechanisms of cancer will continue to contribute to our understanding of the molecular pathogenesis of cancer and in the evaluation of environmental risks. By understanding the molecular pathogenesis of cancer, we can relate oncogenic or epigenetic events occurring in the rodent to changes that occur in human cancer, and make conclusions about the potential human cancer risk of certain compounds, as well as helping to develop new strategies for the diagnosis and treatment of various different types of human cancer.

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Table 1

Summary of genetic mutations in NTP molecular pathology studies.

Organ	Chemical	Species/strain	Tumor type	Genetic mutation	Ancillary testing
Large intestine	o-nitrotoluene	mouse/B6C3F1	Cecal carcinoma	K-Ras p53 Catnb	IHC: CK7+, CK20−, B-catenin+, Cyclin D+, P53+ (Sills *et al.* 2004)
Lung	cumene	mouse/B6C3F1	AB adenoma/carcinoma	K-Ras p53 p16 LOH	microarray:↑ERK-MAP kinase, ↑HDACs, ↑genes associated with invasion, metastasis, anti-apoptosis, angiogenesis ↓tumor suppressor genes, genes associated with inhibition of migration, invasion, and proliferation (Hong *et al.* 2008)
Lung	1,3-Butadiene chloroprene	mouse/B6C3F1	AB adenoma/carcinoma	K-Ras LOH	(Ton *et al.* 2007)
Lung	3-azido-3-deoxythymidine (AZT)	mouse/Swiss (CD-1)	AB adenoma/carcinoma	K-Ras p53	(Hong *et al.* 2007a)
Lung	Ethylene oxide	mouse/B6C3F1	AB adenoma/carcinoma	K-Ras	(Hong *et al.* 2007b)
Liver	Methyleugenol Oxazepam Diethanolamine	mouse/B6C3F1	HCA, HCC, HB	Catnb	IHC: Cyclin D+, nuclear B-catenin, loss of E-cadherin Western blot: ↑cyclin D1, B-catenin deletion (Devereux *et al.* 1999, Hayashi *et al.* 2003, Iida *et al.* 2003)
Harderian gland	Ethylene oxide	mouse/B6C3F1	Papillary cystadenoma/carcinoma	K-Ras	(Hong *et al.* 2007b)
Uterus	Ethylene oxide	mouse/B6C3F1	Uterine carcinoma	K-Ras	(Hong *et al.* 2007b)
Brain	chloroprene	mouse/B6C3F1	glioma and neuroblastoma	p53 K-Ras p16 LOH	(Ton *et al.* 2007)
Skin	8-methoxypsoralin (8-MOP)	mouse/HRA/Skh	Squamous cell carcinoma	p53	IHC: p53+, PCNA+ (Lambertini *et al.* 2005)
Mesothelium	o-nitrotoluene Bromochloracetic acid	Rat/F344	Mesothelioma		microarray: dysregulation of Igf, p38 MAPK, Wnt/B-catenin, and integrin signaling pathways (Kim *et al.* 2006)