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Strain-Dependent Differences in Susceptibility to Lung Cancer in Inbred Mice Exposed to Mainstream Cigarette Smoke

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Abstract

It is becoming increasingly clear that genetic susceptibility is an important host factor determining the effects of exposure to a number of airborne particles and gases. Although numerous studies have identified a genetic component for spontaneous pulmonary tumor development and for chemically-induced lung cancer (e.g., urethane) in mice, a systematic examination of murine interstrain differences in response to cigarette smoke inhalation has not been conducted. We addressed this research gap by examining the strain distribution pattern of lung cancer in 9 inbred strains of mice exposed to 258 mg/m³ mainstream cigarette smoke for 5 months followed by 4 months of rest. Lung tumors were enumerated on fixed-lungs visualized at low magnification and on serial step sections examined microscopically. With the low magnification examination, we observed statistically significant increases in the number of lung tumors in cigarette smoke-exposed A/J and the genetically-related A/HeJ mice ($p < 0.05$). While fewer tumors were identified by the microscopic enumeration method, it confirmed that significant increases in lung tumors occurred only in A/J and A/HeJ mice exposed to cigarette smoke ($p < 0.05$). Thus, as predicted by epidemiologic studies and animal experiments using chemically-induced lung cancer models, these findings suggest that genetic host factors play a significant role in the pulmonary tumorigenic response of mice to mainstream cigarette smoke.

Keywords

mouse; lung cancer; genetic susceptibility; cigarette smoke

INTRODUCTION

Lung cancer is a devastating disease that affects over 200,000 individuals in the U.S. each year [1]. The number of affected individuals throughout the world is, of course, several times greater and it has been estimated that 1.2 million people die worldwide from lung cancer each year [2]. The prognosis for patients diagnosed with lung cancer is often poor and dependent on a number of factors including the stage at which the disease is diagnosed and the responsiveness of the tumor to treatment. Thus, it is important that preventive approaches to lung cancer be identified.

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Inhalation of the combustion products of tobacco, either in the form of mainstream cigarette smoke or environmental tobacco smoke, is the leading cause of lung cancer, in addition to being the cause of several other major human diseases. Approximately 85% of all lung cancer cases have a link to cigarette smoke exposure [1]. Logically, reduction or elimination of exposure to cigarette smoke would bring broad and important improvements to public health and this approach has been widely advocated. In the absence of a ban on tobacco products and the poor performance of smoke cessation programs in many populations, another approach to reduce the morbidity and mortality of lung cancer is to develop preventive approaches that target the cellular and molecular pathways by which inhalation of cigarette smoke produces lung cancer. Understanding the mechanisms which lead to cigarette smoke-induced lung cancer could thus lead to significant improvements in lung cancer prevention and possibly also treatment.

There has been increasing evidence that genetic host factors play a significant role in the inter-subject variability in response to inhaled particles and gases. Evidence for a genetic contribution to the susceptibility to lung cancer is suggested by the fact that only a fraction of cigarette smokers, approximately 15%, develop lung cancer [3]. Genetic susceptibility to cigarette smoke and chemically-induced lung cancer has been demonstrated in both epidemiologic and animal toxicology studies. Epidemiologic studies have shown that a variety of DNA polymorphisms are associated with lung cancer risk. These polymorphisms occur in genes encoding for phase I and II detoxifying enzymes, DNA repair genes, cell cycle control genes, and in tumor suppressor genes and oncogenes [4]. Animal studies have presented evidence that host genetic factors are involved in the tumorigenic response of the lung to individual chemical carcinogens. Using quantitative trait linkage analysis, investigators have identified specific chromosomal loci which are strongly associated with susceptibility or resistance to lung tumor induction in mice treated with ethyl nitrosourea, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or urethane, or in a 2-stage 3-methylcholanthrene/butylated hydroxytoluene model [5,6,7,8].

Although lung tumors can be induced at high incidence in mice treated with certain chemical carcinogens, only few tumors are generally produced in the lungs of mice chronically exposed to mainstream cigarette smoke [9]. Yet, work by Witschi and colleagues [10,11] has provided strong evidence that cigarette smoke does induce substantial numbers of lung tumors in A/J inbred mice when they are allowed to recover for an extended period of time following the chronic exposure. Recently, other investigators have demonstrated that chronic exposure (30 months) to mainstream cigarette smoke produces lung neoplasms in approximately 45% of B6C3F1 mice compared to 10% of control mice [12]. Furthermore, based upon accumulating evidence in other murine models of cancer susceptibility, the low carcinogenic response to cigarette smoke observed in most mouse strains could very likely be the result of genetic insensitivity among the large variety of inbred mouse strains available to date. A systematic examination of murine interstrain differences in lung tumor response to cigarette smoke inhalation has not been conducted - only a few relevant inbred strains have been tested [11, 13,14]. We have directly addressed this research gap by examining differences in lung tumor response among nine inbred strains of mice exposed to mainstream cigarette smoke using the exposure-recovery protocol of Witschi and colleagues [15].

MATERIAL AND METHODS

Animals

Female mice (18 to 25 grams, The Jackson Laboratory, Bar Harbor, ME) were acclimatized to the animal housing facility for a minimum of 2 weeks prior to the start of the exposure protocol. The animals were maintained in a climate-controlled room with a 12-hour light/dark cycle in an AALAC-accredited animal facility and housed in polycarbonate cages with corn cob

bedding and free access to food and water except during exposure to air or cigarette smoke. Individually coded transponder chips (Bio Medic Data Systems, Seaford, DE) were inserted subcutaneously in all mice for identification purposes during this chronic study.

The following ten strains of mice were used: A/J, A/HeJ, AKR, BALB/C, C3H/HeJ, C57BL/6, CAST/Ei, DBA, SWR, and 129/Svi (The Jackson Laboratory, Bar Harbor, ME). Twenty mice of each strain were exposed to cigarette smoke and 10 to 20 mice were exposed to filtered air (Table 1). Body weights were determined monthly and at study termination. Fifteen of the twenty groups of mice that were exposed to air or cigarette smoke had one or more animals removed during the 9 month protocol due to unexpected incidental morbidity and mortality. This included AKR mice, which were all euthanized at approximately 6 months into the study due to signs of leukemia, and some CAST/Ei mice that were extremely difficult to handle. Therefore, AKR and some of the CAST/Ei animals were not included in the analysis. There was no excess mortality associated with the cigarette smoke exposure in any of the strains used, although some inbred strains lost weight during the exposure phase of the study. This study was approved by the Institutional Care and Use of Animals Committee of the NYU School of Medicine.

Cigarette smoke exposure

Strain susceptibility to induction of lung tumors by cigarette smoke was examined using the following exposure protocol developed by Witschi et al [15]. Mice were exposed to filtered air or mainstream cigarette smoke (target concentration 250 mg/m³ total smoke particulates, TSP) for 5.5 hours per day, 5 days per week for 5 months. Exposures were not conducted on federal or religious holidays and these missed exposure days were added at the end of the 5 month exposure period. During the first week, mice were exposed to increasing concentrations of cigarette smoke each day to permit acclimatization to the cigarette smoke. After the 5 months exposure period, the animals were housed for an additional 4 months without treatment to allow tumor development.

Mainstream cigarette smoke was generated from unfiltered 1R1 reference cigarettes (Tobacco and Health Research Institute, Lexington, KY) using a Baumgartner-Jaeger CSM 2070 cigarette smoke generation system (CH Technologies, Westwood, NJ) using the ISO puff profile [16]. Cigarettes were refrigerated until the day prior to use at which time they were conditioned to 55% ± 5% humidity and room temperature. The 10 strains were exposed to cigarette smoke or filtered air using three Laskin-type whole body exposure chambers while housed in solid bottom polycarbonate cages with wire mesh tops on one of three wire mesh shelves per chamber. The physical location of the cages in the chambers was changed (front to back and shelf level to shelf level) on a weekly basis to compensate for any potential differences in exposure concentrations within the exposure chamber. The mice did not have access to food and water during exposure. The exhaust from the cigarette smoke chambers passed through a pre-filter and HEPA filter unit followed by a heated catalytic converter (Johnson Matthey, Wayne, PA) to remove both particles and gases before emission into the environment.

The airborne mass concentration in the two cigarette smoke exposure chambers was gravimetrically measured at least every 2 hours by collecting particles on Pallflex filters (EMFAB TX40HI20-WW, Pall Corporation, East Hills, NY). Carbon monoxide (CO) was also determined hourly using a CO monitor (Model CO-220, Everett, WA) which was calibrated with multi-point standards prepared in a mylar gas bag using dilutions of a 1000 ppm CO gas standard. The mean mass particle and carbon monoxide concentrations in the cigarette smoke exposure chambers were 258 ± 64 mg/m³ (± SD) and 205 ± 66 ppm, respectively, over the 5 month exposure period. Temperature and relative humidity in the chambers, measured daily, were 74 ± 3 ° Fahrenheit and 43 ± 17%, respectively. Analysis of

airborne metal concentrations was determined on additional Teflon filter samples (Gelman Teflo, 37 mm, 0.2µm pore, n = 3) which were analyzed by x-ray fluorescence (EX-6600-AF, Jordan Valley) according to the methods presented by Maciejczyk and Chen [17] and a representative sample is presented in Table 2.

Tumor Enumeration

At the end of the nine month study (five months exposure and four months recovery), the mice were euthanized with an overdose of pentobarbital and exsanguination. The thorax was opened and the lungs were inflated with Tellyesniczky's fluid at a constant pressure of 25 cm H₂O. This fixative was used because it makes the lung translucent so that tumors and other structural features inside the lobes are grossly visible. The trachea was tied off to maintain lung inflation and the lungs were removed from the thorax and stored in fixative. Tumor presence was evaluated by two methods. First, tumors were visually counted on the Tellyesniczky's fixative-cleared lungs at low magnification by a single observer. The lungs were then embedded in paraffin and six step sections (5 µm thick) of each lung lobe were made at 200 µm intervals and stained with H&E. Tumors were identified by light microscopic examination by a single pathologist (MCB) and classified as adenomas or carcinomas using standard criteria for alveologenic tumors in mice [18,19,20,21,22]. The largest diameter of each tumor was measured with an eyepiece micrometer. Foci of hyperplasia were also enumerated by light microscopic examination in the same set of six step sections per lung lobe.

Statistics

Lung tumor data were statistically evaluated in terms of multiplicity (mean number of tumors per animal, including non-tumor bearing animals), incidence (number of animals with tumors over the total number of animals), tumor size (in mm); multiplicity, incidence, and size of hyperplastic lesions were evaluated separately. The independent factors of strain and exposure (cigarette smoke and air) were evaluated with a two-factor ANOVA for 3 end-points: tumor multiplicity, hyperplasia multiplicity, and mean tumor size per animal. In a post-hoc test, Welch's corrected unpaired t test was used to evaluate intra-strain differences in tumor multiplicity, hyperplasia multiplicity, and mean tumor size per animal comparing air-versus cigarette smoke-exposed mice. Because we hypothesized that cigarette smoke would cause an increase in tumors in comparison to air exposure, one-tailed t tests were utilized. Fischer's exact test was used for intra-strain comparison of tumor incidence in air-versus cigarette smoke-exposed mice. All data were analyzed with InStat and Prism statistical software (Graphpad Software, San Diego, CA).

RESULTS

Exposure to cigarette smoke resulted in strain-dependent increases over controls in lung tumor multiplicity (tumors/animal) (Table 1). Using a two-factor ANOVA, both strain and exposure had statistically significant effects on outcome with both the macroscopic and the microscopic method of tumor enumeration. Evaluated by the macroscopic method, significant increases in lung tumor multiplicity were observed in smoke-exposed mice of the A/J and A/HeJ strains (both $p < 0.05$), and this effect nearly reached statistical significance in the DBA strain ($p = 0.06$) and to a lesser extent in BALB/C mice ($p = 0.09$). When evaluated at the light microscopic level, significant increases in lung tumor multiplicity were observed only in A/J and A/HeJ mice (both $p < 0.05$).

The ability to discern lung tumors was dependent on both the enumeration method and the strain. For example, very few DBA and BALB/C mice exposed to cigarette smoke had tumors according to the microscopic evaluation, whereas these strains, particularly BALB/C mice, had lung tumors using macroscopic enumeration. Although more tumors were observed using the

macroscopic enumeration method compared to the microscopic method (approximately 3-fold for A/J and A/HeJ mice, Table 1), the ratio of tumors/animal (multiplicity) in cigarette smoke-exposed compared to air-exposed mice was very similar for the macroscopic and microscopic methods in the 3 strains that were shown to actually have tumors by the microscopic enumeration (i.e., the microscopic and macroscopic air/cigarette smoke ratios were 0.8 and 0.9, respectively, for SWR mice; 2.9 and 2.7, respectively, for A/J mice; and 1.9 and 2.0, respectively, for A/HeJ mice). In addition, the correlation coefficient for tumors/animal comparing the macroscopic and microscopic methods for all A/J and A/HeJ mice was 0.71 ($p < 0.0001$).

The observed increases over controls in multiplicity of lung tumors induced by cigarette smoke in A/J and A/HeJ mice were not accompanied by increases in tumor incidence or mean tumor size (Table 3). Hyperplastic lung lesions were statistically increased in cigarette smoke-exposed mice of the A/J strain, but not the A/HeJ strain or other strains (Table 4). Unexpectedly, hyperplasia multiplicity was significantly greater in C57BL/6 mice exposed to air than in those exposed to cigarette smoke, although no tumors were observed during the microscopic evaluation of serial lung sections. Spontaneous lung tumors were observed in some strains of air-exposed mice, but the number was dependent on the method used to enumerate tumors. As shown in Table 1, spontaneous tumors were observed in the lungs of air animals for all strains when tumors were evaluated by the macroscopic method, but the histopathologic evaluation of lungs demonstrated some spontaneous tumors only in air-exposed A/J, A/HeJ, SWR, BALB/C, and DBA mice.

Spontaneous broncho-alveolar adenomas in air-exposed mice and cigarette smoke-induced broncho-alveolar adenomas were morphologically indistinguishable. No attempt was made to distinguish tumors originating from type II alveolar cells and tumors of Clara cell origin [23]. A few tumors that had characteristics consistent with adenocarcinoma were observed in a couple of air-exposed mice of the SWR and A/J strains and in smoke-exposed mice of the A/J and DBA strains. These tumors had clear evidence of locally invasive growth in combination with increased pleomorphism compared to adenomas (fig a). Adenomas, in contrast, were well delineated, compressing surrounding tissue to a variable degree, and typically displayed a monomorphic cellular phenotype (fig b). However, some adenomas were situated within a larger area of hyperplasia and were less well delineated (fig c), whereas others had areas of increased cellular atypia sometimes bordering on a morphology consistent with adenocarcinoma (fig d), but invasive growth was not apparent in these tumors. There were also a couple of bronchiolar adenomatous papillomas which occurred in an air-exposed A/HeJ mouse and in a smoke-exposed SWR mouse. These lesions were exophytic adeno-papillary tumors that projected into a bronchiolar lumen (fig e).

Discussion

In the present study, we identified considerable inter-strain differences in the susceptibility of nine mouse strains to induction of lung tumors by main-stream cigarette smoke inhalation. These inter-strain differences in tumor response strongly suggest, but do not definitively prove, that genetic factors play a role in cigarette smoke-induced lung cancer susceptibility in mice. Examination of the ability of cigarette smoke to induce lung tumors in mice has provided quite variable results in past research. In general, mice have appeared to be relatively insensitive to the induction of tumors by chronic cigarette smoke exposure [9,24], although a recent set of life-time exposure studies in rats and mice has demonstrated a significant and consistent increase in tumors [25,12]. While a portion of this variability might be explained by inter-laboratory variation, cigarette type (e.g., filtered versus unfiltered), the use of environmental versus mainstream cigarette smoke, and exposure protocol (e.g., concentration), we demonstrate here that strain differences can also considerably influence the induction of lung

tumors in mice by cigarette smoke inhalation. Our observation of an increase in tumors in cigarette smoke-exposed A/J mice confirms the findings by other investigators [15,26]. The other inbred mouse strains used in this study, however, have not been used extensively by other research groups and, at maximum, only 2 or 3 inbred strains have been compared by others for cigarette smoke-induced lung cancer [11]. Analysis of tumor responses in multiple inbred strains has been successfully used in identifying chromosomal loci containing susceptibility gene(s) involved in lung tumorigenesis produced by chemical carcinogens [8,27,28] and such studies should pave the way for more rapid identification of genes contributing to the genetic susceptibility to cigarette smoke-induced lung cancer.

In the present study, we compared the macroscopic enumeration of tumors with the microscopic evaluation of serial step-sections taken across all lobes at 200 μ m intervals. Using microscopic evaluation, the mean tumor counts/animal were lower than those observed macroscopically, but the ratio of tumors/animal in cigarette smoke- versus air-exposed A/J, A/HeJ, and SWR mice was nearly identical for both methods. This suggests that both enumeration methods are reliable measures of cigarette smoke-induced lung tumors, although the macroscopic enumeration method may overestimate tumor counts, particularly in some strains (e.g., BALB/C). As previously examined by [29], unidentified factors may have accounted for the absolute differences in the counts between the 2 methods. For example, the macroscopic enumeration (performed on Tellyesniczky's- and ethanol-fixed lungs using a 4 $\times$ magnifying lens) may have included counts of lung lesions other than lung tumors, such as foci of inflammation and clusters of pigment-laden alveolar macrophages that frequently occur in smoke-exposed mice of some strains but not others (unpublished observations). It is difficult-to-impossible to distinguish these lesions from small tumors by macroscopic examination. The microscopic evaluation, meanwhile, could have underestimated the number of lung tumors if tumors smaller than the step section interval (200 μ m) were routinely missed. However, we did a pilot evaluation on a subset of lungs using paraffin sections made at 50 and 100 μ m step intervals and up to 12 step sections per lung. A step section interval of 200 μ m and 6 sections per lung resulted in a maximally detected number of tumors; smaller intervals and more sections per lung did not significantly increase the tumor counts. Moreover, the smallest observed tumors were 0.1 mm (100 μ m) in diameter, but these were few, and most tumors were 0.2 mm in diameter or larger. Thus, even the vast majority of 'smallest' tumors were not likely to be missed in our approach of examining 6 step sections made at 200 μ m intervals.

In addition to A/J mice, increases in lung tumor multiplicity (tumors/animal) have been reported for BALB/C and SWR inbred mice [11], Swiss albino mice [13], and B6C3F1 [12] mice exposed to environmental or mainstream cigarette smoke. We did not observe a similar effect in SWR mice after exposure to mainstream cigarette smoke and there was only a modest increase, which did not reach statistical significance, in microscopic tumors/animals in BALB/C mice in our study. Our study also determined that A/HeJ mice develop significantly more lung tumors/animal after exposure to mainstream cigarette smoke than did air-exposed mice. Because of the close genetic relatedness of A/J and A/HeJ mice, this finding suggests that the 2 strains may share functional nucleotide polymorphism(s) (e.g., C5 gene which plays a central role in the complement pathway activation), gene copy number, or gene deletions that contribute to cigarette smoke-induced lung cancer.

Although we have previously reported strain-dependent differences in the emphysematous and pulmonary hypertension response to mainstream cigarette smoke exposure [30], there are limitations to the interpretation of strain-dependent differences in lung tumor induction in the present study. The majority of the tumors were adenomas, and as discussed by other investigators [11,31], adenomas themselves are not invasive tumors but may progress to adenocarcinomas. Indeed, in this study we found evidence for such progression observing small adenomas within hyperplastic lesions, areas with increased cellular atypia within adenomas,

and a few adenocarcinomas that contained areas that were rather cellularly monomorphic and morphologically similar to adenomas. We did not, however, observe morphologic or size differences in the tumors observed in the 9 strains examined in this study. In addition, the use of a single gender, female, in the present study, limits the extrapolation of our positive findings to male mice of the same strains. All but one of the environmental tobacco smoke exposure studies by Witschi and colleagues (2005) [11] were done with male A/J mice. Ongoing work in our lab, however, has demonstrated that female A/J mice have a greater increase in tumors/animal after mainstream cigarette smoke exposure than do male A/J mice (data not shown). Interestingly, epidemiologic studies have provided evidence that female smokers may be at higher risk than men for cigarette smoke-induced lung cancer [32], although this has not been a consistent association in both case-control and cohort epidemiology studies [33]. Finally, A/J mice develop adenomas spontaneously so that it is unclear if the cigarette smoke-induced increase in adenomas is due to new tumors or enhanced growth promoted by cigarette smoke exposure. However, if predominantly enhanced growth, not new tumor development, was induced by cigarette smoke exposure, one would expect an increase in the size, not the increased multiplicity which we observed. Thus, cigarette smoke exposure may have induced new tumors in this study. Individual components of cigarette smoke are complete carcinogens and mainstream cigarette smoke contains both tumor initiating and promoting factors, so cigarette smoke-induced increases in tumors/animal in A/J mice may be a combination of complete carcinogenesis and tumor promotion. To disentangle tumor initiation, tumor promotion, and complete carcinogenesis in cigarette smoke-induced lung tumorigenesis in mice is very difficult. One way to approach this issue could be to compare the results of cigarette smoke exposure with those to lung tumorigenesis induced in A/J mice by a known tumor-initiating agent alone.

The purpose of this study was to delineate a strain distribution pattern in the lung tumor response of inbred mouse strains exposed to cigarette smoke. This is the first step in identifying sensitive and resistant strains for use in classic genetic model studies [34] to identify chromosomal regions, and eventually genes, that play a role in the complex trait of susceptibility to cigarette smoke-induced lung cancer. A number of quantitative trait loci (QTL) have been reported for murine lung cancer induced by chemical carcinogens including components of cigarette smoke, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and urethane [5,6,7,8]. As with environmental cigarette smoke [11,26], A/J mice have been found to be a highly susceptible strain in terms of chemically carcinogen-induced lung cancer. Although these studies have made important contributions to the understanding of genetic susceptibility factors in lung cancer models, our study is the first to examine the strain dependent response in a large number of inbred mouse strains exposed to the complex mixture of particles and gases that make up cigarette smoke. One goal of this study was to identify a strain of mice that was susceptible to mainstream cigarette smoke-induced lung cancer, but did not have a significant spontaneous tumor rate. We observed a general trend for cigarette smoke-exposed mice to have a greater number of tumors/animal than air-exposed mice in several strains (increased in 7 of 9 strains and 5 of 9 strains for the macroscopic and microscopic evaluations, respectively; Table 1). However, statistical significance was reached only in A/J and A/HeJ mice probably because of the weak carcinogenicity of cigarette smoke and the limited number of animals in each exposure group (10 to 20 mice). Nonetheless, these findings suggest that our approach holds potential in identifying host genetic susceptibility factors in mice exposed to cigarette smoke, the most important cause of lung cancer throughout the world. With this knowledge and using classic genetic model studies in mice [34], host genetic susceptibility factors can be examined in linkage analysis studies of crosses between strains of mice sensitive and resistant to cigarette smoke-induced lung cancer. These cigarette smoke studies will be important because, to date, QTL studies have used individual chemical carcinogens and may not be predictive of the genetic susceptibility factors responsible for the lung cancer induced by the complex mixture of particles and gases present in cigarette smoke.

In addition, *in silico* computational models may also prove valuable in understanding genetic susceptibility in inbred mouse strains [35,36]. For example, identification of the functional sequence differences between A/J and A/HeJ mice and the other inbred strains could yield important insight into which gene(s) contribute to the strain-dependent difference in susceptibility to cigarette smoke-induced lung tumors.

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References

1. Dubey S, Powell CA. Update in lung cancer 2007. *Am J Respir Crit Care Med* 2008;177(9):941–946. [PubMed: 18434333]
2. Youlten DR, Cramb SM, Baade PD. The International Epidemiology of Lung Cancer: geographical distribution and secular trends. *J Thorac Oncol* 2008;3(8):819–831. [PubMed: 18670299]
3. Spitz MR, Wei Q, Dong Q, Amos CI, Wu X. Genetic susceptibility to lung cancer: the role of DNA damage and repair. *Cancer Epidemiology, Biomarkers & Prevention* 2003;12:689–698.
4. Risch A, Plass C. Lung cancer epigenetics and genetics. *Int J Cancer* 2008;123(1):1–7. [PubMed: 18425819]
5. Dragani TA, Manenti G, Pierotti MA. Polygenic inheritance of predisposition to lung cancer. *Ann Ist Super Sanita* 1996;32(1):145–150.
6. Fijnenman RJA, de Vries SS, Jansen RC, Demant P. Complex interactions of new quantitative trait loci, *Sluc1*, *Sluc2*, *Sluc3*, and *Sluc4*, that influence the susceptibility to lung cancer in the mouse. *Nat Genet* 1996;14:465–467. [PubMed: 8944028]
7. Festing MFW, Yang A, Malkinson AM. At least four genes and sex are associated with susceptibility to urethane-induced adenomas in mice. *Genet Res* 1994;64:99–106. [PubMed: 7813906]
8. Malkinson AM, Radcliffe RA, Bauer AK. Quantitative trait locus mapping of susceptibilities to butylated hydroxytoluene-induced lung tumor promotion and pulmonary inflammation in CXB mice. *Carcinogenesis* 2002;23(3):411–417. [PubMed: 11895855]
9. Henry CJ, Kouri RE. Chronic inhalation studies in mice. II Effects of long-term exposure to 2R1 cigarette smoke on (C57BL/Cum x C3H/AnfCum)F1 mice. *J Natl Cancer Inst* 1986;77(1):203–212. [PubMed: 3459913]
10. Witschi HP, Espiritu I, Maronpot RR, Pinkerton KE, Jones AD. The carcinogenic potential of the gas phase of environmental tobacco smoke. *Carcinogenesis* 1997;18:2035–2042. [PubMed: 9395199]
11. Witschi HA. A/J mouse as a model for lung tumorigenesis caused by tobacco smoke: strengths and weaknesses. *Exp Lung Res* 2005;31(1):3–18. [PubMed: 15765916]
12. Hutt JA, Vuilleminot BR, Barr EB, Grimes MJ, Hahn FF, Hobbs CH, March TH, Gigliotti AP, Seilkop SK, Finch GL, Mauderly JL, Belinsky SA. Life-span inhalation exposure to mainstream cigarette smoke induces lung cancer in B6C3F1 mice through genetic and epigenetic pathways. *Carcinogenesis* 2005;26(11):1999–2009. [PubMed: 15944214]
13. De Flora S, Izzotti A, D'Agostini F, Bennicelli C, You M, Lubet RA, Balansky RM. Induction and modulation of lung tumors: genomic and transcriptional alterations in cigarette smoke-exposed mice. *Exp Lung Res* 2005;31(1):19–35. [PubMed: 15765917]
14. Hecht SS. Carcinogenicity studies of inhaled cigarette smoke in laboratory animals: old and new. *Carcinogenesis* 2005;26(9):1488–1492. [PubMed: 15930027]
15. Witschi H, Espiritu I, Dance ST, Miller MS. A mouse lung tumor model of tobacco smoke carcinogenesis. *Toxicol Sci* 2002;68(2):322–330. [PubMed: 12151628]

16. ISO 3308, 1991E ISO 3308. (1991E). Routine analytical cigarette smoking machine—Definitions and standard conditions, Geneva, Switzerland, International Organization for Standardization.
17. Maciejczyk P, Chen LC. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice, VIII, Source-related daily variations in in vitro responses to CAPs. *Inhal Toxicol* 2005;17(4–5):243–253. [PubMed: 15804942]
18. Dixon D, Horton J, Haseman JK, Talley F, Greenwell A, Nettesheim P, Hook GE, Maronpot RR. Histomorphology and ultrastructure of spontaneous pulmonary neoplasms in strain A mice. *Exp Lung Res* 1991;17(2):131–155. [PubMed: 1646706]
19. Hamm JT, Yee S, Rajendran N, Morrissey RL, Richter SJ, Misra M. Histological alterations in male A/J mice following nose-only exposure to tobacco smoke. *Inhal Toxicol* 2007;19(5):405–418. [PubMed: 17365046]
20. Hahn FF, Gigliotti AP, Hutt JA, March TH, Mauderly JL. A review of the histopathology of cigarette smoke-induced lung cancer in rats and mice. *Int J Toxicol* 2007;26(4):307–313. [PubMed: 17661221]
21. Foley JF, Anderson MW, Stoner GD, Gaul BW, Hardisty JF, Maronpot RR. Proliferative lesions of the mouse lung: progression studies in strain A mice. *Exp Lung Res* 1991;17(2):157–168. [PubMed: 2050022]
22. Yao R, Wang Y, Lubet RA, Ming Y. Differentially expressed genes associated with mouse lung tumor progression. *Oncogenomics* 2002;21(37):5814–5821.
23. Sato T, Kauffman SL. A scanning electron microscopic study of the Type II and Clara cell adenoma of the mouse lung. *Lab Invest* 1980;43(1):28–36. [PubMed: 7392573]
24. Finch GL, Nikula KJ, Belinsky SA, Barr EB, Stoner GD, Lechner JF. Failure of cigarette smoke to induce or promote lung cancer in the A/J mouse. *Cancer Lett* 1996;99(2):161–7. [PubMed: 8616820]
25. Mauderly JL, Gigliotti AP, Barr EB, Bechtold WE, Belinsky SA, Hahn FF, Hobbs CA, March TH, Seilkop SK, Finch GL. Chronic inhalation exposure to mainstream cigarette smoke increases lung and nasal tumor incidence in rats. *Toxicol Sci* 2004;81(2):280–292. [PubMed: 15213336]
26. D'Agostini F, Balansky RM, Bennicelli C, Lubet RA, Kelloff GJ, De Flora S. Pilot studies evaluating the lung tumor yield in cigarette smoke-exposed mice. *Int J Oncol* 2001;18(3):607–615. [PubMed: 11179494]
27. Liu P, Wang Y, Vikis H, Maciag A, Wang D, Lu Y, Liu Y, You M. Candidate lung tumor susceptibility genes identified through whole-genome association analyses in inbred mice. *Nat Genet* 2006;38(8):888–895. [PubMed: 16862160]
28. Wang M, Zhang Z, Zhang Z, Vikis H, Yan Y, Wang Y, You M. Fine mapping and candidate gene analyses of pulmonary adenoma resistance 1, a major genetic determinant of mouse lung adenoma resistance. *Cancer Res* 2007;67(6):2508–2516. [PubMed: 17363568]
29. Tillmann T, Kamino K, Dasenbrock C, Kohler M, Morawietz G, Campo E, Cardesa A, Tomatis L, Mohr U. Quality control of three methods for lung tumorigenesis studies. *Exp Toxicol Pathol* 1999;51(1):99–104. [PubMed: 10048721]
30. Nadziejko C, Fang K, Bravo A, Gordon T. Susceptibility to pulmonary hypertension in inbred strains of mice exposed to cigarette smoke. *J Appl Physiol* 2007;102(5):1780–1785. [PubMed: 17272409]
31. Coggins CR. An updated review of inhalation studies with cigarette smoke in laboratory animals. *Int J Toxicol* 2007;26:331–338. [PubMed: 17661224]
32. Risch HA, Howe GR, Jain M, et al. Are female smokers at higher risk for lung cancer than male smokers?, A case-control analysis by histologic type. *Am J Epidemiol* 1993;138:281–293. [PubMed: 8395141]
33. Neugut AI, Jacobson JS. Women and lung cancer: gender equality at a crossroad? *JAMA* 2006;296(2):218–9. [PubMed: 16835429]
34. Silver, LM. Mouse genetics, Concepts and applications. Oxford Univ Press; NY: 1995.
35. Grupe A, Germer S, Usuka J, Aud D, Belknap JK, Klein RF, Ahluwalia MK, Higuchi R, Peltz G. In silico mapping of complex disease-related traits in mic. *Science* 2001;292(5523):1915–1918. [PubMed: 11397946]
36. Pletcher MT, McClurg P, Batalov S, Su AI, Barnes SW, Lagler E, Korstanje R, Wang X, Nusskern D, Bogue MA, Mural RJ, Paigen B, Wiltshire T. Use of a dense single nucleotide polymorphism map for in silico mapping in the mouse. *PLoS Biol* 2004;2(12):e393. [PubMed: 15534693]

1. .

A–C: Hyperplasia; D–F: Adenoma; G–I: low grade adenocarcinoma. Bar = 50 μ m (panel A), 100 μ m (panels B–F, H, & I), 200 μ m (panel G). Arrows: Panel C: area of early adenoma formation; panel E: area of increased cellular atypia; panel G: Invasion in blood vessel; panels H & I: areas of mononuclear inflammatory cells. Panel H is higher power image of box in panel G.

Table 1

Tumor multiplicity (mean $\pm$ SEM) for lung tumors evaluated by the macroscopic and microscopic methods (see Methods section)

Strain/Exposure	n	Tumors/animal		Tumors/animal	
		(Macroscopic)	SEM	(Microscopic)	SEM
A/J AIR	18	1.06	.27	0.39	0.15
A/J CS	20	3.10*	.48	1.05*	0.29
A/HeJ AIR	20	1.45	0.33	0.30	0.11
A/HeJ CS	18	2.68*	0.49	0.61*	0.15
C3H AIR	10	0.50	0.24	0	NA
C3H CS	18	0.89	.27	0	NA
DBA AIR	19	0.90	.26	0.05	0.05
DBA CS	18	1.56	.32	0	NA
CAST AIR	7	0.43	.32	0	NA
CAST CS	17	1.00	.38	0	NA
C57 AIR	9	1.30	.5	0	NA
C57 CS	20	1.05	.19	0.10	0.07
BALB AIR	10	2.50	.42	0.10	0.11
BALB CS	19	3.32	.44	0.21	0.10
129 AIR	10	2.10	.82	0	NA
129 CS	19	2.37	.29	0.05	0.06
SWR Air	5	3.00	1.2	0.80	0.42
SWR CS	19	2.42	.35	0.74	0.13

NA = not applicable as there was only 0 or 1 tumor in the group.

* Within a strain, cigarette smoke value was significantly different than the air group value ($p < 0.05$) in a one-tailed Welch's corrected t test.

Table 2Trace element concentrations in the cigarette smoke exposure atmosphere ($\mu\text{g}/\text{m}^3$, mean $\pm$ SD) *

Element	Mean	SD
S	265.2	35.8
Cl	513.9	52.6
K	486.0	38.9
Ca	781.9	59.3
Ti	104.1	2.8
Fe	20.5	2.7
Zn	133.0	10.1
Br	12.4	2.2
Rb	7.7	1.7
Sr	5.6	1.6
In	187.8	32.4
Sb	91.6	14.1

* Other metals such as V, Cr, Mn, Co, Ni, Se, and Cd were detected but are not presented because the measured concentrations were 3 times below the uncertainty level as determined by measurement of blank filters.

Table 3
Tumor Incidence and Size Identified by the Microscopic Evaluation of Serial Sections

Strain/Exposure	n	Animals w/tumors	% Animals w/tumors	Mean Tumor Size (mm)	SEM
A/J AIR	18	6	33%	0.90	0.23
A/J CS	20	12	60%	0.61	0.07
A/HeJ AIR	20	6	30%	0.63	0.18
A/HeJ CS	18	10	56%	1.23	0.71
C3H AIR	10	0	0%	0	NA
C3H CS	18	0	0%	0	NA
DBA AIR	19	1	5%	0.70	NA
DBA CS	18	0	0%	0	NA
CAST AIR	7	0	0%	0	NA
CAST CS	17	0	0%	0	NA
C57 AIR	9	0	0%	0	NA
C57 CS	20	2	10%	0.40	0.14
BALB AIR	10	1	10%	0.50	NA
BALB CS	19	4	21%	0.93	0.3
129 AIR	10	0	0%	0	NA
129 CS	19	1	5%	0.20	NA
SWR Air	5	3	60%	0.68	0.36
SWR CS	19	13	68%	0.52	0.06

NA = not applicable as there were no tumors in the group.

Table 4

Hyperplasia multiplicity (n/group is the same as in Table 3)

Strain/Exposure	Hyperplastic lesions/animal	
	Mean	SEM
A/J AIR	0.39	0.12
A/J CS	0.95*	0.25
A/HeJ AIR	0.40	0.16
A/HeJ CS	0.44	0.17
C3H AIR	0.20	0.14
C3H CS	0.17	0.09
DBA AIR	0.21	0.10
DBA CS	0.11	0.08
CAST AIR	0	NA
CAST CS	0	NA
C57 AIR	0.67	0.35
C57 CS	0.05	0.05
BALB AIR	0.20	0.14
BALB CS	0.26	0.11
129 AIR	0	NA
129 CS	0.32	0.14
SWR Air	0.20	0.22
SWR CS	0.53	0.16

NA = not applicable as there were no regions of hyperplasia in the group.

* Within a strain, cigarette smoke value was significantly different than the air group value ($p < 0.05$) in a one-tailed Welch's corrected *t* test.