Outbred CD-1 mice carry the susceptibility allele at the pulmonary adenoma susceptibility 1 (Pas1) locus

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CD-1 is the outbred mouse line most often used in toxicology and carcinogenicity bioassays. A literature survey revealed a relatively high (21.8%) incidence of spontaneous lung tumors in these mice, and a susceptibility to lung tumorigenesis induced by vinyl chloride, styrene or benzene inhalation that is not seen in B6C3F1 or C57BL/6 mice, or in rats and hamsters. As the pulmonary adenoma susceptibility 1 (Pas1) locus on distal chromosome 6 (8) is the major determinant of genetic susceptibility to lung tumorigenesis in mice, we analyzed CD-1 mice for genetic polymorphisms of the Kras2 and Pthlh genes, which are tightly linked with the Pas1 locus. From 95 to 98% of CD-1 mice carried the susceptibility allele at the Pas1 locus either at homozygosity or heterozygosity, providing a molecular genetic explanation for the high susceptibility of CD-1 mice to spontaneous and chemically induced lung tumorigenesis. These results may have implications for the risk assessment of chemicals in humans using experimental animals that display strain-specific lung tumorigenicity.

Introduction

Mouse inbred strains show a natural variation in susceptibility to tumorigenesis in different organs (1). The incidence of spontaneous tumors correlates with strain susceptibility to tumorigenesis in the same target organ and complicates the evaluation of carcinogenicity bioassays, especially when the spontaneous tumor incidence is high and variable (2–4).

Recent insights from mouse genetics have explained the high susceptibility of some mouse strains to the development of certain tumors. For example, B6C3F1 mice, which are commonly used in the National Toxicology Program (NTP) carcinogenicity bioassay, carry hepatocellular tumor susceptibility loci that underlie their high susceptibility to chemically induced hepatocarcinogenesis (5,6), while A/J mice, commonly used in medium-term lung tumor bioassays (7), inherit the susceptibility allele at the pulmonary adenoma susceptibility 1 (Pas1) locus on distal chromosome 6 (8).

CD-1, an outbred mouse line derived from the original colony of Swiss mice started in 1926 (9), are commonly used in toxicology and in chemical carcinogenicity bioassays.

However, until now, no genetic analysis of tumor susceptibility loci carried by these mice has been conducted.

Herein, we describe our literature search indicating a relatively high incidence of spontaneous lung tumors and a high susceptibility to chemically induced lung tumorigenesis in CD-1 mice. Analysis of genetic markers linked to the Pas1 locus revealed the Pas1 susceptibility allele in a high percentage (95–98%) of CD-1 mice.

Materials and methods

Literature survey

A computerized literature search was carried out to identify studies of CD-1 mice reporting spontaneous lung tumor incidence (Table I) and studies of chemical carcinogenicity induced by inhalation exposure, in which both CD-1 mice and other mouse strains or rodent species were treated with the same chemical (Table II). Data of male and female mice from the spontaneous tumor incidence studies were compared statistically by using the Fisher’s exact test and the SAS (SAS Institute, Cary, NC) software.

Mice and DNA extraction

Eighty-eight male and 31 female outbred CD-1 mice, retired breeders of ~1 year of age, were purchased from Charles River (Calco, Italy). Genomic DNA from these mice was extracted from frozen spleens of these mice using the Genomix kit and an automatic DNA extractor (Talent, Trieste, Italy).

Genetic marker analysis

Genetic markers were amplified by 40 cycles of PCR (55°C annealing temperature), using the primers reported in Table III. Kras2-37 was detected by agarose gel electrophoresis. Kras2_SNP397, Ddh3n38 and Pthlh markers were genotyped by allele-specific oligonucleotide (ASO) hybridization; briefly, an aliquot of the PCR mix was checked on agarose gel, and the remaining amount was denatured in 0.4 NaOH/25 mM EDTA at room temperature and spotted onto a nylon membrane by a Biomek 2000 robot (Beckman). Replica filters

Table I. Lifespan incidence of spontaneous lung tumors in untreated CD-1 mice

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<th>Study</th>
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<th>Percent incidence</th>
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were pre-hybridized for 2 h at 55°C and hybridized at 45°C for 3 h in a solution containing 50 mM Tris±HCl pH 7.5, 2 mM EDTA, 5 denhardt’s, 3 M TMAC (Sigma), 1% SDS, 200 μg/ml salmon sperm DNA, and 32P-end-labeled 15mer oligonucleotides containing the polymorphic base at the eighth nucleotide (Table III). Washes were performed at 37°C and at room temperature in 2× SSC. Hybridization signals were detected using high-performance autoradiography film.

**Results**

CD-1 mice are genetically susceptible to lung tumorigenesis

Analysis of literature data on spontaneous lung tumors in CD-1 mice revealed a mean tumor incidence of 21.8%, with a range of 8.8±61.1% (Table I). The control groups in the reported studies include a total of 7913 CD-1 mice, thus constituting a representative sample of the CD-1 population. Male mice showed a significantly higher mean lung tumor incidence than females (25.2 versus 18.2%, P < 0.001, Fisher’s exact test) (Table I). Lung tumors that developed in CD-1 mice showed histological characteristics of lung adenomas or carcinomas typical of mouse lung tumors (10,12).

CD-1 mouse susceptibility to chemically induced lung tumorigenesis was compared with that of other mouse strains and rodent species in the context of chemical inhalation-induced lung carcinogenicity in which CD-1 mice, B6C3F1 or other mouse strains (C57BL/6) genetically resistant to lung tumorigenesis or other rodent species were treated with the same chemical (Table II). In three studies that applied multiple doses of vinyl chloride (VC) in CD-1 mice (13–15), a dose-related lung tumor response was observed. The lowest VC dose that produced a significant increase in lung tumor incidence was 50 p.p.m.. VC treatment at 50 p.p.m. also induced lung tumors in A/J but not in B6C3F1 mice (16,17). Both CD-1 and B6C3F1 mice developed angiosarcomas (primarily of soft tissues) and mammary tumors after VC treatment at the same dose (50 p.p.m.) and schedules, but only CD-1 mice developed lung tumors (17). B6C3F1 mice were not treated with higher VC doses. VC was not carcinogenic in the lungs of a total of 4741 inhalation-exposed rats, at doses up to 30 000 p.p.m. or in 805 inhalation-exposed hamsters at doses up to 10 000 p.p.m. (13–15,17–19).

Styrene inhalation at doses as low as 20 p.p.m. induced lung carcinogenesis in CD-1 mice, whereas it was not lung-carcinogenic in rats treated with 15-fold higher doses (20,21). Finally, benzene inhalation induced lung tumors in CD-1 mice but not in C57BL/6 mice treated at the same dose (300 p.p.m.) in the same experiment (22) (Table II).

CD-1 mice carry the Pas1<sup>+</sup> allele

CD-1 mice were analyzed for four genetic markers defining the Pas1 locus. Kras2-37, a 37-bp length polymorphism located in the second intron of the Kras2 gene, was detected in 113 (95%) of 119 CD-1 mice, as the single 37-bp repeat carried by A/J mice, whereas only six (5%) CD-1 mice were homozygous for the double 37-bp repeat carried by the C57BL/6 J strain (Table IV). The frequency of the A/J-related Kras2 allele was 0.81 in CD-1 mice.

Similar observations were made for the non-coding Kras2_SNP397 polymorphism, located in exon 4B of the Kras2 gene (GenBank acc. no. U76426) ~26 kb distant from the Kras2-37 polymorphism (http://www.ensembl.org); 112 of
118 (94.9%) CD-1 mice carried the A nucleotide of the A/J strain, whereas only six (5.1%) mice were homozygous for the C nucleotide carried by the C57BL/6 J mice (Tables III and IV). Genotypes overlapping those of the above markers were observed for the D6Int38 marker, a silent polymorphism located within the gene AK001783 ~55 kb distance from the Kras2-37 polymorphism. D6Int38 showed significant linkage disequilibrium with lung tumor susceptibility in inbred strains (not shown), and CD-1 mice carried the same allele as that in the A/J strain (Table IV, Figure 1A and B).

Genotyping of an SNP coding polymorphism of the Pthlh gene, resulting in PthlhPro and PthlhThr alleles, revealed the PthlhThr allele carried by A/J mice in 112 of 114 (98.2%) CD-1 mice, resulting in allele frequency of 0.86. Only two (1.8%) mice carried the PthlhPro allele at homozygosity (Tables III and IV).

None of the genotypes showed significant deviation from the Hardy–Weinberg equilibrium (not shown).

Discussion

CD-1 mice are characterized by a relatively high incidence of spontaneous lung tumors (mean 21.8%). This percentage is lower than the lifetime incidence of spontaneous lung tumors in A/J mice (80–90%) (23) or SWR/J mice (37–50%) (24) but similar to that of BALB/c mice (22.3–29.4%) (25,26) and higher than that of B6C3F1 mice (8.6–11.4%) (27,28) or of F344 rats (1.0–3.7%) (28). CD-1 mice are also particularly susceptible to chemically induced lung tumorigenesis, as certain chemicals show lung carcinogenic effects in CD-1 mice but not in other rodent species. For example, VC inhalation induces lung tumors in CD-1 and A/J mice (16), but not in B6C3F1 mice (17), rats (13–15,18,19), or hamsters (14,17) (Table II). Styrene by inhalation is lung-carcinogenic in CD-1 mice but not in rats treated with 15-fold higher doses (Table II). Benzene inhalation is carcinogenic for the lung of CD-1 mice but not for that of C57BL/6 J mice (Table II). Benzene by gavage was lung-carcinogenic in B6C3F1 mice but not in rats (29). However, none of those studies were aimed at testing strain differences in dose response, and it remains possible that higher dose levels of these three chemicals induce lung tumors in genetically resistant strains and species.

In most of the studies above, only lung tumor incidence was evaluated as the tumor phenotype, most likely leading to an underestimation of the genetic differences in susceptibility. Analysis of lung tumor multiplicity might have provided a much more powerful indicator of susceptibility (30). In any case, those studies provide evidence that CD-1 mice are more susceptible to lung tumorigenesis as compared with B6C3F1 and C57BL/6 J mice, rats, or hamsters, as these latter strains and species did not show a lung tumorigenesis response at doses of chemical carcinogens that are clearly lung-carcinogenic in CD-1 mice.

Although not genetically susceptible to lung tumorigenesis, B6C3F1 mice and rats can develop lung tumors in response to a variety of chemical carcinogens (http://ntp-server.niehs.nih.gov/) (31,32). Hamsters are also responsive to chemical induction of lung tumors (33,34).

Epidemiological studies have reported contrasting evidence regarding human lung cancer risk in VC-exposed workers; however, neither of two recent large studies including more
than 20,000 workers indicated an association between VC exposure and lung cancer risk (35,36). For styrene, there is limited evidence for a possible increased risk of lymphatic and haematopoietic neoplasms but not lung tumors (37). Benzene is a well-known leukemogenic agent in rodents as well as in humans; a marginally significant excess for lung cancer was observed in benzene-exposed workers in a single study (38,39).

Previous studies have identified the *Pas1* locus as the major determinant of lung tumor susceptibility in mice, without carcinogen-specific effects (8,40). *A/J*, *SWR/J* and BALB/c mice carry the *Pas1I* allele, whereas *C57BL/6 J*, *C3H/He*, and their cross, B6C3F1, mice carry the *Pas1I* allele (41). *Pas1* locus activity has not been studied in rats or hamsters, but no strong strain differences in lung tumor susceptibility have been reported in those species. In humans, studies in first-degree relatives of lung cancer probands have established a link between genetic factors and a significant risk of lung cancer, although cigarette smoking remains the major risk factor for this cancer (42,43). There is some evidence suggesting that genetic markers located in the human *Pas1* homologous region affect risk and prognosis of lung adenocarcinoma (44,45). However, establishing any role for the *Pas1* locus in humans awaits identification of a *Pas1* gene.

Nonetheless, present evidence indicates that a cluster of genetic polymorphisms in five genes located in a 452 kb region around *Kras2* have segregated in *Pas1I* mice (46 and not shown). The *Kras2* 37 bp polymorphism, as well as the three other SNPs herein genotyped, are tightly associated with genetic predisposition to lung tumorigenesis and *Pas1I* allele status in inbred strains (41,47,48).

CD-1 mice can be considered an island population, and no comprehensive analysis of genetic polymorphisms in these mice has yet been conducted. In a small-scale study on iso-zyme variations, average heterozygosity was 0.067, i.e. slightly lower than those of feral mice (0.034–0.11) (9), but precise estimation of CD-1 genetic heterozygosity awaits extensive analysis of DNA marker polymorphisms in comparison with feral mice. We expect that certain chromosomal regions are fixed at homozygosity in the colony, whereas a significant degree of polymorphism is maintained in other regions. Our finding that the outbred CD-1 mice carry at high frequency (0.80±0.86) the same *D6Int38*, *Kras2* and *Pthlh* alleles carried by the *A/J* and other *Pas1I* mouse strains appears to account for the high genetic susceptibility of CD-1 mice to lung tumorigenesis. Indeed, only 2–5% of CD-1 mice were homozygous for the *Pas1I* allele and thus genetically resistant to lung tumorigenesis. The high frequency of the *Pas1I* allele in CD-1 mice provides a molecular genetic explanation for their high spontaneous incidence of lung tumors and high susceptibility to chemically induced lung tumorigenesis. Two highly susceptible *Pas1I* strains (SWR/J and NGP/N) were also derived from the original colony of Swiss mice used to derive CD-1 mice (29,41,50), suggesting a high frequency of the *Pas1I* allele in the original colony and consistent with the present findings.

While the *Pas1* susceptibility allele appears to underlie the high susceptibility to lung tumorigenesis of CD-1 mice, this allele does not affect strain susceptibility to carcinogenicity in other organs. Indeed, CD-1 and B6C3F1 mice, rats and hamsters are equally susceptible to VC-induced angiosarcomas (13–15,17). Moreover, C57BL/6 mice were more susceptible than CD-1 mice to induction of Zymbal gland tumors by benzene (22), and B6C3F1 mice and F344 rats treated with benzene by gavage developed tumors at multiple sites (29).

Although CD-1, BALB/c and A/J mice all carry the same *Pas1I* allele (41), the incidence of spontaneous lung tumors in CD-1 mice is lower than that of A/J mice (80–90%) (23) and similar to that of BALB/c mice (22.3–29.4%) (25,26). The *Pas1I* allele causes a dominant lung tumor susceptibility trait, with allele dosage effects, when inherited by the C57BL/6 J and C3H/He strains (8,51). However, the *Pas1I* allele is also subject to down-modulation by pulmonary adenoma resistance loci (*Par*), which can completely abrogate or partially inhibit susceptibility to lung tumorigenesis (51). The lower lung tumor incidence in BALB/c mice as compared with the *A/J* strain may reflect the presence of *Par2* and *Par4* loci in BALB/c mice (52). Several inbred strains carry *Par* loci (51) and it is possible that CD-1 mice do as well, partially inhibiting the effects of the *Pas1I* allele. In principle, it should be possible to fine-map the putative *Par* loci carried by these outbred mice, using linkage disequilibrium analysis (41,46).

Contrasting results in different mouse strains or in different rodent species in analyses of chemicals for their risk of lung cancer induction underscore the need to understand the strain-specific mechanism of lung carcinogenesis in experimental animals and its possible relevance for humans. While species-specific differences in metabolism or pharmacokinetics of the tested chemicals have been invoked to explain differences in the tumorigenic response (53,54), genetic variations might also represent a major determinant of the specific tumor response to chemicals. Indeed, each rodent strain carries a specific set of susceptibility and resistance alleles at cancer modifier loci, which affect sensitivity to chemical carcinogenesis (1,55,56).

To date, several mouse cancer modifier genes have been identified, whereas characterization of polygenic inheritance of cancer predisposition in rats and humans lags far behind that in mouse models. Availability of the complete nucleotide sequences of the mouse and human genomes might shed light on the genetic mechanisms underlying strain and species differences in response to chemical carcinogens. Although the genetic mechanisms of these differences remain unknown, we suggest considering the genetic susceptibility of strain and species in the risk assessment of chemicals to pursue a scientifically based extrapolation of carcinogenic risk of chemicals from rodents to humans.

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**References**


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