A Cyp2a polymorphism predicts susceptibility to NNK-induced lung tumorigenesis in mice

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Lung tumors from smokers as well as lung tumors from mice exposed to tobacco carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK), often carry mutations in K-ras, which activates downstream-signaling pathways such as PI3K/AKT/mTOR pathway. Mice with genetic deletion of one of three isoforms of AKT were used to investigate the role of AKT in mutant K-ras-induced lung tumorigenesis in mice. Although deletion of Akt1 or Akt2 decreased NNK-induced lung tumor formation by 90%, deletion of Akt2 failed to decrease lung tumorigenesis in two other mouse models driven by mutant K-ras. Genetic mapping showed that Akt2 was tightly linked to the cytochrome P450 Cyp2a locus on chromosome 7. Consequently, targeted deletion of Akt2 created linkage to a strain-specific Cyp2a5 polymorphism that decreased activation of NNK in vitro. Mice with this Cyp2a5 polymorphism had decreased NNK-induced DNA adduct formation in vivo and decreased NNK-induced lung tumorigenesis. These studies support human epidemiological studies linking CYP2A polymorphisms with lung cancer risk in humans and highlight the need to confirm phenotypes of genetically engineered mice in multiple mouse strains.

Introduction

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) is the most potent and prevalent nitrosamine procarcinogen in cigarette smoke and has been used extensively to study tobacco carcinogen-induced lung tumors in mice (1). Cytochrome P450 CYP2A enzymes cause \( \alpha \)-hydroxylation of NNK to form reactive intermediates, which can interact with DNA to produce mutations (1). All P450 enzymes require cytochrome P450 reductase (CPR) as an electron donor. Genetic deletion of CPR in mouse lung decreases NNK-induced lung tumorigenesis, suggesting that lung tumors result primarily from lung-specific generation of carcinogenic NNK metabolites (2). Consistent with this, genetic polymorphisms that decrease enzymatic activity of human CYP2A13, the predominant CYP2A isoform in lung, correlate with a 4- to 5-fold decrease in lung cancer risk in smokers (3,4).

The A/J mouse strain is the most commonly used strain for studies of lung tumorigenesis due to its high propensity for both spontaneous and carcinogen-induced lung tumor formation. K-ras is the main lung tumor susceptibility allele in A/J mice (5). Many genetically engineered mice have been generated in strains other than A/J. To make genetically engineered mice susceptible to lung tumorigenesis, they have been backcrossed to A/J (2,6). Here, we have used this approach to investigate the requirement for each of the three Akt isoforms in NNK-induced lung carcinogenesis.

Although the results for Akt1 or Akt3 deletion were consistent across mouse models (7), the results with Akt2 deletion were inconsistent. Akt2+/- mice developed 90% fewer NNK-induced lung tumors than their +/- littermates. However, in a genetic model of mutant K-ras-induced lung tumorigenesis, latent-activatable K-ras (LA2) (8), Akt2+/- mice did not show any decrease in lung tumor formation. Akt2+/- mice also did not develop fewer lung tumors in response to urethane, which also induces mutant K-ras-dependent lung tumors (9). Loss of Akt2 did not decrease systemic elimination of NNK but did decrease NNK-induced \( \alpha \)-methylguanamine (\( \alpha \)-mG) DNA adducts and decreased in vitro bioactivation of NNK by lung microsomes. Despite the apparent association with loss of Akt2, the decrease in NNK-induced lung tumorigenesis and associated lung metabolism and DNA adducts in Akt2+/- mice is attributable to the very tightly linked Cyp2a5 locus. The Akt2+/-- Cyp2a5 is derived from the 129 strain used to generate the genetically engineered mice. This strain carries a polymorphism in Cyp2a5 that leads to a one amino acid change in the protein relative to the A/J strain. In the absence of any Akt2 alteration, the A/J polymorphism segregates with NNK-induced lung tumor susceptibility and correlates with increased NNK metabolism by lung microsomes. These data not only clarify the minimal involvement of Akt2 in lung carcinogenesis in mouse models but also support a cause and effect relationship between Cyp2a polymorphisms that alter lung CYP2A activity and tobacco-induced lung cancer.

Materials and methods

Mouse husbandry and treatment

All experiments were done under an NIH approved animal study protocol. NIH is an AAALAC-certified facility. Mice were housed in plexiglas cages with water and NIH31 diet provided ad libitum. Akt2+/- mice (10) on a C57BL/6 background were a generous gift from Dr. Morris Birnbaum. These mice were backcrossed twice with A/J mice, which are susceptible to NNK-induced lung tumorigenesis induced by NNK treatment. Progeny from subsequent Akt2+/- intercrosses carried two copies of the wild-type A/J K-ras allele and are referred to as A/J-N2. Akt2+/- (A/J-N2) were also backcrossed for six additional generations to A/J to generate mice in a nearly pure A/J background (designated A/J-N8). Akt2+/- (A/J-N2 and A/J-N8) intercrosses were used to generate littermates for carcinogen-induced lung tumorigenesis studies. Mice were injected intraperitoneally with 3 weekly doses of NNK (Toronto Research Chemicals, Toronto, Canada) at 100 mg/kg beginning at 6 weeks of age. Urethane was administered intraperitoneally as a single dose (1000 mg/kg). A/J-N2 Akt2+/- mice were also crossed with K-ras\(^{LA2}\) mice (8) to generate K-ras\(^{LA2}\) mice with varying Akt2 genotypes. At 22 weeks of age for mice on carcinogen studies or 8 weeks of age for K-ras\(^{LA2}\) studies, mice were killed by cervical dislocation and lungs were inflated and fixed with 10% neutral-buffered formalin. The following day, the lobes of the lungs were separated and surface lung tumors were counted and measured using a dissecting microscope fitted with a micrometer. Data were analyzed using Prism (GraphPad Software, San Diego, CA) using unpaired t-tests.

Genotyping and marker identification

Genotypes were determined by polymerase chain reaction (PCR) on DNA from tail clips. Briefly, 5 mm of the tail tip was lysed overnight in 100 ml of 0.1 M Tris, pH 8, 5 mM ethylenediaminetetraacetic acid, 0.2% sodium dodecyl sulfate, 200 mM NaCl and 100 mM proteinase K. The following day, 300 ml of water was added, and samples were boiled for 10 min before PCR, for which 1 ml was used for each sample. For Akt2 genotyping, primers used were: wt, 5’-GCACCTTCGGCAAGGTCA-3’; common, 5’-GCTGTTGCTCCTAATAACTCTGTG-3’; mutant, 5’-GCATGTCCTCCAGCTG-CCCTT-3’. Fragment sizes were 237 bp for the Akt2 wt and 170 bp for the Akt2 mutant. K-ras allele strain contribution was determined by PCR for K-ras_37 (http://www.informatics.jax.org/searches/polymorphism.shtml).

To detect strain contribution of the Cyp2a5 allele in Akt2 carcinogenesis studies, PCR on Cyp2a5 was performed using primers

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Mouse chromosome 7 marker list including Cyp2a, Akt2 and microsatellite alleles in mouse strains contributing to the genetic background in Akt2−/− and +/- mice

<table>
<thead>
<tr>
<th>Marker</th>
<th>Allele A/J</th>
<th>Allele B6</th>
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</thead>
<tbody>
<tr>
<td>Cyp2a5</td>
<td>6.5 bp</td>
<td>117 bp</td>
</tr>
<tr>
<td>Akt2</td>
<td>6.5 bp</td>
<td>117 bp</td>
</tr>
<tr>
<td>D7Mit267</td>
<td>11 bp</td>
<td>182 bp</td>
</tr>
<tr>
<td>D7Mit230</td>
<td>24.5 bp</td>
<td>107 bp</td>
</tr>
<tr>
<td>D7Mit231</td>
<td>53.0 bp</td>
<td>116 bp</td>
</tr>
<tr>
<td>D7Mit71</td>
<td>65.2 bp</td>
<td>112 bp</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>153M</td>
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</table>

**Strain contribution of the indicated markers among chromosome 7 in the Akt2−/+ or Akt2−/− mice.**

**Location in bp along mouse chromosome 7 of the indicated genes or markers.**

**Strain contribution of the indicated markers along chromosome 7 in the Akt2−/+ or Akt2−/− mice.**

**The 129 allele is Akt2−/− since it is derived from the original targeted embryonic stem cells used to generate Akt2−/− mice.**

**D7Mit267 was used to differentiate between A/J and 129 or C57BL/6 chromosome 7.**

**D7Mit230, D7Mit238 and D7Mit71 were used to determine the strain contribution of chromosome 7 in Akt2−/+ mice in the pure A/J background.**

<table>
<thead>
<tr>
<th>Chemicals and reagents</th>
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<tbody>
<tr>
<td>4-Hydroxy-1-(3-pyridyl)-1-butanone (HPB) and 4-(methylnitrosamino)-1-(3-pyridyl)-N-oxide (NNK-N-Oxide) were purchased from Toronto Research Chemicals (Ontario, Canada); sodium bisulfite and reduced sodium bisulfite (NADPH) were purchased from Sigma–Aldrich (St Louis, MO). 4-Oxo-4-(3-pyridyl) butanal (OPB) was obtained from Santa Cruz Biotechnology. The CYP2A antibody (sc-67165; Santa Cruz Biotechnology). Blots were processed and scanned and signal intensity quantified on an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) as described previously (6).</td>
</tr>
</tbody>
</table>

**Immunoblot analysis**

Lung or liver tissue was homogenized with a dounce homogenizer in 125 mM Tris, pH 6.8, 20% glycerol and 4% sodium dodecyl sulfate. Lysates were spun at 9000 g for 10 min at 25°C and the supernatant collected. 25 μg of protein was boiled in 5% β-mercaptoethanol, size separated on 12–16% Bis–Tris polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were incubated with antibodies to pan-CYP2A (sc-3214; Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (CP01; Oncogene Research Products, Boston, MA) or GAPDH (sc-67165; Santa Cruz Biotechnology). Blots were processed and scanned and signal intensity quantified on an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) as described previously (6).

**Results**

The role of Akt2 in mutant K-ras-induced lung tumorigenesis was investigated using carcinogen-driven and genetic models. Akt2−/− mice were used as a lung tumor-resistant C57BL/6 background were crossed twice with lung tumor susceptible A/J mice to generate Akt2+/− mice carrying two copies of the A/J K-ras gene and lung tumor susceptibility. Akt2−/− and Akt2+/− mice in this A/J-N2 background developed 89 and 83% fewer NNK-induced lung tumors than their +/+ littermates, respectively (Figure 1A). However, Akt2−/−/K-rasA2 mice did not develop fewer lung tumors than their Akt2+/+K-rasA2 littermates (Figure 1B). Since NNK metabolism was thought to have a possible role in the differences observed with the NNK tumor model, a third tumor model was used, with which urethane induces K-ras mutations leading to lung tumors. However, urethane metabolism is metabolized by different mechanisms than that for NNK; therefore, the urethane tumor model is useful for delineating the effects of NNK-specific metabolism on lung tumorigenesis. Akt2−/− mice developed more urethane-induced lung tumors than their +/+ littermates (Figure 1C). These data suggested that Akt2 is not a general requirement for mutant K-ras-mediated lung tumorigenesis but may be specific for the tumorigenic response to NNK.

Because Akt2 is tightly linked to the Cyp2a locus that encompasses Cyp2ar4, Cyp2ar5 and Cyp2al2, we hypothesized that genetic deletion of Akt2 might alter CYP2A expression. CYP2A protein levels were determined in liver and lung by immunoblotting. The CYP2A antibody detects all three CYP2A isoforms, which are all expressed in the liver, whereas CYP2A5 is the only CYP2A isoform expressed in the lung (12). No differences in CYP2A expression were observed for lungs or liver among Akt2−/−, +/- and +/+ mice (Figure 2), which shows that Akt2 loss does not affect levels of CYP2A protein expression in the liver or levels of CYP2A protein expression in the liver.
To evaluate the loss of Akt2 on NNK absorption and elimination, A/J-N2 mice were treated with NNK, and plasma was taken at defined time points to measure levels of NNK and its procarcinogenic metabolite, 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanol. Up to 4 h after injection, levels of NNK and 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanol did not differ between Akt2+/− and +/+ mice (Figure 3A). This suggested that systemic metabolism of NNK was not altered in Akt2+/− mice. However, analyses of DNA from lung tissues from these same mice showed that Akt2+/− mice contained fewer NNK-induced O\(^{6}\)-mG DNA adducts than Akt2+/+ mice (Figure 3B, supplementary Table S1 is available at Carcinogenesis Online). O\(^{6}\)-mG DNA adducts were not detected in tissues from saline-treated mice (data not shown).

NNK is metabolized in tissues to form both inactive compounds as well as reactive intermediates that can cause DNA mutations. In vitro metabolism of NNK was investigated in microsomes isolated from lung and liver tissues of Akt2+/−, +/+ and +/+ mice in a nearly pure (N8) A/J background (supplementary Fig. S1 is available at Carcinogenesis Online). Akt2+/− lung microsomes showed decreased rates of NNK metabolism to OPB, a trapped reactive intermediate, compared with Akt2+/+ microsomes. Akt2+/− lung microsomes showed intermediate rates (Figure 4A). There were no differences in lung microsomal metabolism of NNK to HPB or N-oxide or in metabolism to any product in the liver microsomes (supplementary Table S2 is available at Carcinogenesis Online). The differing results for the formation of the three NNK metabolites in the lungs are consistent with the fact that tumorigenesis correlates with metabolism to OPB, but not to HPB or N-oxide, and with the notion that OPB represents the major carcinogenic metabolite of NNK (1). In that regard, the different NNK metabolites are known to be preferentially formed by different CYP2A enzymes. Although CYP2A5 is expressed in both liver and lung, certain other CYP2 enzymes capable of metabolizing NNK, such as CYP2A4, show liver-specific expression.

Consistent with the in vitro data, decreased levels of NNK-induced O\(^{6}\)-mG DNA adducts were found in A/J-N8 Akt2−/− and Akt2+/− lungs (Figure 4B). In contrast, there was no difference in the levels of liver O\(^{6}\)-mG adducts among the genotypes (supplementary Table S3 is available at Carcinogenesis Online). In the A/J-N8 background, Akt2−/− and Akt2+/− mice still developed fewer NNK-induced lung tumors (Figure 4C), confirming the role of the Akt2 locus in NNK-induced tumorigenesis. The reproducibility of the A/J-N2 data in the A/J-N8 mice suggests that the locus-surrounding Akt2 alone is responsible for the differences observed in NNK metabolism in vitro, and NNK-induced DNA adduct formation and tumorigenesis in vivo.

Lung-specific metabolism of NNK was correlated with NNK-induced DNA adducts and NNK-induced tumorigenesis in Akt2 mouse. Thus, potential effects of Akt2 deletion on NNK metabolism were investigated. The cytochrome P450 enzymes of the CYP2A subfamily are known to metabolize NNK (13) and CYP2A5 is the only mouse CYP2A expressed in the lung (12). Therefore, CYP2A5 was investigated further in Akt2−/− mice. Akt2 deletion did not alter CYP2A5 expression in the lung or CYP2A (4, 5 and 12) expression in the liver (Figure 2). Akt2 and Cyp2a5 genes are tightly linked on mouse chromosome 7 at 28.4 and 27.6 Mb respectively. A polymorphism in Cyp2a5 between 129 and A/J strain which carries one Cyp2a5 coding region polymorphism relative to the A/J strain; the polymorphism results in one amino acid change (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=snp Detail&key=4165313). The strain contribution of the Cyp2a5 gene was further verified using PCR/restriction enzyme digestion (as described in Materials and Methods), and the Cyp2a5 polymorphism was verified in Akt2+/− and Akt2+/+ mice by sequencing a PCR product encompassing the polymorphism. All Akt2+/− mice carried the 117V allele, whereas all Akt2+/+ mice carried the 117A allele (data not shown).

Since Akt2−/− mice carry both Akt2 deletion and CYP2A5−/−, the Akt2 deletion was a confounding factor in the analysis of the effect of CYP2A5 polymorphism on lung tumorigenesis. Therefore, additional studies were undertaken using A/J and 129 strains in the absence of any Akt2 alteration. Lung microsomes from 129 mice showed decreased NNK metabolism to OPB compared with the A/J strain (Figure 5A and supplementary Table S4 is available at Carcinogenesis Online). Since CYP2A5 is the only CYP2A in lung microsomes, this suggested that the altered NNK metabolism was due to the A117V polymorphism.

NNK-induced tumorigenesis was then compared in A/J and 129 strains and in F1, F2 and F3 crosses. Pure 129 mice did not develop NNK-induced lung tumors, whereas A/J mice developed an average of 24 tumors per mouse (Figure 5B). The F1 cross of A/J and 129 developed less than one lung tumor per mouse. F1 were crossed with A/J to generate F2 mice, and F2 mice were again crossed with A/J to generate F3 mice. Both F2 and F3 mice carried either two copies of the A/J Cyp2a5 or one each from A/J and 129. Collectively, F2 and F3 mice developed an average of eight lung tumors per mouse. However, when either F2 or F3 mice were stratified based on Cyp2a5 allele, mice with one 129 and one A/J allele had significantly fewer lung tumors than mice with two A/J alleles (Figure 5B). Therefore, the 129 Cyp2a5 allele segregates with relative NNK-induced lung tumor resistance.
Fig. 2. *Akt2* deletion does not affect CYP2A protein levels in the lung or liver. Tissue homogenates (25 μg protein each) were analyzed on western blots probed with a pan-CYP2A antibody. Each lane is from one mouse liver or lung. Gapdh or β-actin was analyzed as a loading control. γ, CYP2A expression was normalized to Gapdh or β-actin levels and averaged for each tissue by genotype (males and females combined). Differences were not significant for all pairwise comparisons for both lung and liver.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relative CYP2A expression</th>
<th>Act2+/+</th>
<th>Act2+-</th>
<th>Act2+-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td></td>
<td>1.04 ± 0.18</td>
<td>1.18 ± 0.19</td>
<td>0.96 ± 0.13</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>1.01 ± 0.08</td>
<td>1.21 ± 0.18</td>
<td>1.19 ± 0.14</td>
</tr>
</tbody>
</table>

Fig. 3. *Akt2−/−* mice do not show altered systemic clearance of NNK but show decreased levels of NNK-induced DNA adducts. Three mice per group were treated with a single intraperitoneal injection of NNK at 200 mg/kg. (A) Plasma was obtained at the indicated time points for analysis of NNK and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol levels. (B) At 240 min, lung tissues were flash frozen prior to analysis for O6-mG DNA adducts. Values shown represent means ± SD.

Fig. 4. Mouse lung microsomes exhibit decreased NNK bioactivation with decreasing Akt copy number, which is correlated with decreased levels of NNK-induced O6-mG DNA adducts in vivo and decreased NNK-induced lung tumor multiplicity. All mice were in an A/J-N8 background. (A) Rates (means ± SD) of OPB formation in vitro by lung microsomes, six mice per group. (B) Abundance of O6-mG DNA adducts (means ± SD) in lungs at 4 h after a 100 mg/kg NNK injection, six mice per group. (C) Number (means ± SEM) of surface lung tumors per mouse at 16 weeks after the first of 3 weekly doses of 100 mg/kg NNK. Mice per group: +/- (10), +/- (6), +/- (11).
Online) than in Akt2 background (supplementary Figure S2 is available at Carcinogenesis the A/J-N2 background (Figure 1C) and 37% higher in the A/J-N8 129 (5), A129-F1 (4), A129-F2 (31), A129-F3 (15), F2-A (15), F2-H (16), F3-A (3), F3-H (12).

In humans, polymorphisms in CYP2A6, encoding the primary lung-specific cytochrome P450 that metabolizes NNK (16), affects lung cancer risk. In particular, a polymorphism that decreases NNK metabolism by CYP2A13 in vitro decreased lung cancer risk by nearly 5-fold in smokers (3,4). Despite these intriguing human epidemiological studies, there have been no mouse studies showing that polymorphisms in CYP2A2 activity contribute to tobacco carcinogen-induced lung cancer.

Of the 117A/V polymorphisms with NNK metabolic acti-

tion, 117V is found in CYP2A6 (supplementary Figure S3 is available at Carcinogenesis Online), which is expressed primarily in liver, and shows decreased efficiency in the bioactivation of NNK in vitro. In fact, site-directed mutation of CYP2A13 to generate 117V decreases NNK metabolic activation in vitro (14). In lung microsomes, which express CYP2A5, NNK metabolism was decreased in the 129 strain with CYP2A5-117V, compared with the A/J strain with CYP2A5-117A (Figure 5A). This is consistent with a cause and effect relationship between decreased lung-specific metabolism of NNK and resistance to tobacco-induced lung tumors.

Notably, CYP2A5 from the C57BL/6 strain, which is resistant to NNK-induced lung tumorigenesis, carries 117A, as in A/J. Previously, a significant difference in the rates of NNK metabolic activation and formation of O²-mG in the lungs was not found between C57BL/6 and A/J mice (18), which is consistent with the present finding on the association of the 117A/V polymorphisms with NNK metabolic activation in the lungs of 129 and A/J mice. The apparent non-association of the 117A Cyp2a5 allele with lung tumor susceptibility in the B6 mice may be explained by the presence of a carcinogen-independent lung tumor resistant K-ras allele (19).

In contrast to NNK-induced lung tumorigenesis, the number of urethane-induced lung tumors in Ak2+/− mice was 74% higher in the A/J-N2 background (Figure 1C) and 37% higher in the A/J-N8 background (supplementary Figure S2 is available at Carcinogenesis Online) than in Ak2+/- mice. The reason for this increase in susceptibility to urethane-induced lung tumors is not clear. Bioactivation of urethane involves CYP2E1, as Cyp2e1−/− mice exhibit decreased urethane metabolism in vitro and are relatively resistant to urethane-induced lung tumors (20,21). Although CYP2A5 levels were found to be decreased in the liver of Cyp2e1−/− mice (22), it is not yet known whether CYP2A5 can metabolize urethane. Thus, in view of the results presented here, it will be interesting to determine whether CYP2A5 is involved in the bioactivation and subsequent lung tumorigenesis following urethane exposure. If CYP2A5 is involved in urethane metabolism, then the 129-derived CYP2A5 (117V) would appear to confer decreased susceptibility to NNK-induced but increased susceptibility to urethane-induced lung carcinogenesis when compared with the A/J-derived CYP2A5 (117A). It is tempting to speculate that the CYP2A5-117V polymorphism has opposing effects on NNK and urethane metabolism, a situation analogous to the previous report that mutation of 117V to 117A decreased CYP2A5’s coumarin–hydroxylase activity but increased its steroid-15α-hydroxylase activity (23).

The observation that a relatively small difference in NNK metabolic rate can lead to substantial differences in NNK-induced lung tumor incidence mirrors the reported association of the CYP2A13*2 allele, which is characterized by a mild decrease in metabolic activity toward NNK and in gene expression (11,24), with significantly lowered lung cancer incidence in human smokers (3). The disparate differences in lung microsomal NNK bioactivation and in NNK-induced tumor multiplicity between WT and Ak2−/− mice may be explained by the threshold effect in NNK tumorigenesis. This threshold effect might also explain the intermediate response of the Ak2+/− mice in NNK metabolic activation and DNA adduct formation coupled with a profound effect on lung tumor multiplicity (Figure 4). Lower levels of NNK bioactivation may lead to DNA adduct levels that are insufficient to cause lung tumors due to efficient and timely repair. Only metabolic activation leading to adduct levels above this capacity for repair would lead to tumor formation. In A/J mice, it was reported that NNK doses of <2 μmol/mouse (~20 mg/kg, for a single intraperitoneal injection) were only very slightly tumorigenic if at all while lung tumor multiplicity increased dramatically at >3 μmol/mouse (25). This threshold value for lung tumorigenesis by NNK is probably much higher in the 129 strain, due at least in part to the Akt2−/− background (26). This study identified a locus including K-ras, which is not surprising since both spontaneous and urethane-induced lung tumors

Discussion

Decreased metabolism/activation of the tobacco carcinogen, NNK, can confer resistance to NNK-induced lung cancer in mice (2,15). In mice, polymorphisms in CYP2A13, encoding the primary lung-specific cytochrome P450 that metabolizes NNK (16), affects lung cancer risk. In particular, a polymorphism that decreases NNK metabolism by CYP2A13 in vitro decreased lung cancer risk by nearly 5-fold in smokers (3,4). Despite these intriguing human epidemiological studies, there have been no mouse studies showing that polymorphisms in CYP2A2 activity contribute to tobacco carcinogen-induced lung cancer.

Our studies identify a Cyp2a5 polymorphism that correlates with tobacco carcinogen-induced lung cancer risk in 129 and A/J mice. This polymorphism has been described in other strains and can alter metabolism of coumarin (17). In susceptible A/J mice, this polymorphism results in an alanine at amino acid 117 (117A), whereas in the resistant 129 mice, valine is at amino acid 117 (117V). Comparison with human CYP2A enzymes revealed that 117A is found in CYP2A13, the primary lung expressed CYP2A in humans. In contrast, 117V is found in CYP2A6 (supplementary Figure S3 is available at Carcinogenesis Online), which is expressed primarily in liver, and shows decreased efficiency in the bioactivation of NNK in vitro. In fact, site-directed mutation of CYP2A13 to generate 117V decreases NNK metabolic activation in vitro (14). In lung microsomes, which express CYP2A5, NNK metabolism was decreased in the 129 strain with CYP2A5-117V, compared with the A/J strain with CYP2A5-117A (Figure 5A). This is consistent with a cause and effect relationship between decreased lung-specific metabolism/activation of NNK and resistance to tobacco-induced lung tumors.

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In mice, multiple genome-wide association studies have identified loci associated with spontaneous or urethane-induced lung cancer risk. A recent study showed a significant correlation between spontaneous lung tumor incidence and urethane-induced lung tumor multiplicity (26). That study identified a locus including K-ras, which is not surprising since both spontaneous and urethane-induced lung tumors

**Fig. 5.** Lung microsomes from 129 mice show decreased bioactivation of NNK that correlates with decreased NNK-induced lung tumorigenesis. All mice were treated with NNK as described in Methods. A/J, A/J-N8 mice; 129, pure 129X1 mice; F1, A/J × 129; F2, A129F1 × A/J; F3, A129F2 × A/J; A denotes F2 or F3 with two copies of the A/J Cyp2a5 allele; H denotes F2 or F3 with one copy each of the A/J and 129 Cyp2a5 alleles (heterozygous). Mice per group: A/J (5), 129 (5), A129-F1 (4), A129-F2 (31), A129-F3 (15), F2-A (15), F2-H (16), F3-A (3), F3-H (12).

NNK-induced lung tumorigenesis in mice
in mice carry mutant K-ras, a known driver of lung tumorigenesis in mice and humans (8,9,27). NNK-induced lung tumors are dependent on mutant K-ras as well. The finding that a Cyp2a5 polymorphism correlates with NNK-induced lung tumor susceptibility suggests that mouse genome-wide association studies done thus far may have missed at least some critical lung tumor susceptibility loci that have analogous human counterparts. This notion is also highlighted by the recent findings that polymorphisms in nicotine receptors (particularly nAchR5) increase lung cancer risk (28,29,30). NNK, which is derived from nicotine, is known to bind to nAchR (31) but the cause and effect role of this receptor binding in tobacco-induced lung tumorigenesis is unclear.

A Cyp2a5 polymorphism predicting NNK-induced lung tumorigenesis was identified from studies of the role of Akt2 in mutant K-ras-mediated lung tumorigenesis in mice. Although initial studies using NNK suggested that Akt2 was required for lung tumorigenesis, this finding did not hold up with two other models of mutant K-ras-induced lung tumorigenesis. The polymorphism in Cyp2a5 was identified upon investigation of how Akt2 might influence the response to NNK. Although a direct role of Akt2 in the response to NNK has not been ruled out, the linkage of Akt2 to a Cyp2a5 polymorphism clearly is a major contributor to the phenotype observed. These studies highlight the need to utilize multiple mouse models for these studies and provide further evidence that great caution should be exercised when interpreting data using mice with mixed genetic backgrounds, even after extensive backcrossing.

Supplementary material
Supplementary Tables S1–S4 and Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

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References

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