Lung Cancer Susceptibility in Fhit-Deficient Mice Is Increased by Vhl Haploinsufficiency

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Abstract

The FHIT gene plays important roles in cancer development, including lung cancers, in which the Fhit protein is frequently lost. To determine if Fhit-deficient mice exhibit increased susceptibility to carcinogen-induced lung cancer, mice were treated with the pulmonary carcinogen 4-methylnitrosamine-1-3-pyridyl-1-butanone. Wild-type and Fhit-deficient animals did not exhibit significantly different frequencies of lung lesions, but Fhit+/− mice showed significantly increased average tumor volume (1.62 mm³) and multiplicity in tumor-bearing mice, compared with wild-type mice (0.70 mm³). Tumors of Fhit−/− mice were all carcinomas, whereas Fhit+/− mice did not develop carcinomas. To determine if Fhit absence, in combination with deficiency of an additional 3p tumor suppressor, would affect the frequency of tumor induction, we examined the spontaneous and dimethylnitrosamine-induced tumor phenotype of Fhit−/− Vhl+/− mice. Whereas no spontaneous lung tumors were observed in Fhit−/− or Vhl−/− mice, 44% of Fhit−/− Vhl−/− mice developed adenocarcinomas by 2 years of age. Dimethylnitrosamine (6 mg/kg body weight) induced lung tumors (adenomas and carcinomas) in 100% of Fhit−/− Vhl−/− mice and adenomas in 40% of Fhit−/− mice by 20 months of age. Thus, double deficiency in murine homologues of 3p suppressor genes, including haploinsufficiency of Vhl, predisposes to spontaneous and induced lung cancers, showing that Fhit-deficient mice will be useful, in combination with other 3p tumor suppressors, in recapitulating a pattern of lung cancer development similar to the human pattern; such double- or triple-deficient mice will be excellent lung cancer prevention and therapy models. (Cancer Res 2005; 65(15): 6576-82)

Introduction

Lung cancer, the most common human malignancy and major cause of cancer deaths (1), has diverse genetic, biochemical, and histologic characteristics, although the loss of multiple regions of chromosome 3p, harboring a number of known or suspected tumor suppressor genes, occurs in most lung cancers (reviewed in ref. 2). Recently characterized mouse models provide tools to study mechanisms involved in development of genetically uniform lung cancers. For example, a model of lung adenocarcinoma harboring a conditionally activated allele of oncogenic Kras was developed (3) in which recombiant adenovirus expressing Cre recombinase was used to induce Kras expression in the lungs of mice, allowing control of the timing and multiplicity of tumor initiation and characterization of the stages of tumor progression. Another conditional mouse model developed small cell lung cancer (SCLC) through inactivation of Rb1 and Trp53 in lung epithelial cells (4). These are interesting and useful models but do not necessarily develop tumors through alterations to the signal pathways most frequently altered early in development of lung cancers in humans. We have been interested in testing the role of specific tumor suppressors, known to be altered in large fractions of human lung cancers of various histotypes, such as the tumor suppressors identified on the short arm of human chromosome 3, to determine how single or multiple 3p gene alterations affect lung cancer development in mice, as a prelude to studies of prevention and therapy of such mouse tumors.

A number of studies have shown that Fhit, the gene product of the FHIT tumor suppressor gene at 3p14.2, is lost in most human lung cancers reviewed in refs. (5–7). The murine Fhit gene is similar in sequence, location, and fragility to its human homologue (8), suggesting that Fhit-deficient mice might provide a model to study the role of the Fhit pathway in lung cancer development. Fhit-deficient mice, both Fhit−/+ and Fhit−/−, displayed an elevated frequency of a spectrum of spontaneous tumors, but the incidence of lung tumors was not elevated (9). Because Fhit-deficient mice are prone to carcinogen-induced gastric tumors (9, 10) and carcinogen-induced lung neoplasia in mice is a well-documented process, we have studied lung cancer induction in Fhit-deficient mice using two environmental carcinogenic nitrosamines, 4-methylnitrosamino-1-3-pyridyl-1-butanone (NNK) and dimethylnitrosamine.

The FHIT gene and multiple other tumor suppressor genes are located on 3p in man but on several chromosomes in mice. The VHL gene maps to human chromosome 3p25 and is inactivated in most kidney cancers. It is not commonly mutated in lung cancers (11) but 3p25 alleles, including VHL alleles, are lost in >80% of non–small cell lung cancers (NSCLC) studied in the United Kingdom (12). Mice homozygous for deleted Vhl alleles are not viable (13) and mice with conditional knockout of both alleles in liver develop cavernous hemangiomas of the liver (14). Ma et al. (15) used a nonorgan specific approach to make a conditional knockout strain and found hepatic hemangiomas, as well as angiectasis of kidney and other organs. To determine if inactivation of both Fhit alleles in combination with absence of one Vhl allele would enhance susceptibility to lung tumorigenesis, we crossed Fhit and Vhl knockout (Vhl−/−) mice and studied the spontaneous and dimethylnitrosamine-induced tumor phenotypes of the progeny.
Materials and Methods

**Experimental design.** These studies were approved by the University Institutional Animal Care and Use Committee and conducted under the NIH guidelines.

**4-Methylnitrosamino-1-3-pyridyl-1-butanolone treatment.** C57Bl/6j × 129/SvF1) mice (B6129 F1) Fhit+/− and Fhit-deficient male mice (1-3 months of age) received i.p. injections of NNK (Toronto Research Chemicals, Canada) dissolved in PBS, at 200 mg/kg of body weight, every month for 5 months. Mice were sacrificed at 12 to 15 months of age.

**Dimethylnitrosamine treatment.** B6129 female Fhit−/− mice were mated with B6129 Vhl+/−/− mice (13) to obtain double heterozygous Fhit−/−/Vhl−/− mice. The double heterozygous mice were crossed with Fhit+/−/Vhl+/− mice to obtain Fhit+/−/Vhl+/− mice, Fhit−/−/Vhl−/− and Fhit−/−/Vhl−/− mice (2-3 months old) of both sexes received a single i.p. injection of dimethyl-nitrosamine (Sigma, St. Louis, MO) dissolved in water, at 2 or 6 mg/kg of body weight. Dimethylnitrosamine-treated mice were sacrificed at 19 to 20 months of age. Untreated Fhit−/−/Vhl−/− mice were sacrificed at ~25 months of age. Fhit and Vhl mice were genotyped as previously described (10, 13).

**Tumor analysis.** After anesthetization with isoflurane (Ohemda, Madison, WI), mice were sacrificed and autopsied. Lungs were inflated and fixed in buffered formalin, embedded in paraffin, sectioned and stained with H&E for histopathology studies. Lung tumors >0.5 mm in diameter (d) were counted and tumor volume was calculated according to the formula: volume = (π/6) × d³, assuming that all tumors were spherical. This calculation was not possible in the Fhit-Vhl study, due to the highly heterogeneous shape of the tumors. Histopathologic analysis was done by two pathologists and the specimens were described according to the WHO classification (16).

**Immunohistochemistry.** Formalin-fixed and paraffin-embedded tissue sections (4 µm) were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was done by submerging sections in 1 mol/L urea and microwaving for 10 minutes at full power. Endogenous peroxidase activity was quenched using 3% H2O2 in methanol. Sections were blocked in 5% bovine serum albumin (BSA) in PBS and stained at an antibody concentration of 1 µg/mL in 5% BSA in PBS. Antibodies used were anti-Vhl (FL181, Santa Cruz Biotechnology, Santa Cruz, CA) or anti–vascular endothelial growth factor (anti-VEGF, 147, Santa Cruz Biotechnology). Antibody reactivity was developed using a broad-spectrum poly-horseradish peroxidase conjugate and AEC substrate (Zymed Laboratories, South San Francisco, CA). Sections were counterstained with methyl green and mounted in crystal mount permanent mounting solution (Santa Cruz Biotechnology).

**Statistical analysis.** In the NNK study, differences in tumor volumes among mice of the three different genotypes were analyzed using Student’s t test. In the Fhit-Vhl study, differences in tumor incidence among the groups of mice were determined by Fisher's exact test. The same test was applied in the NNK study to determine the significance of differences in pathology of Fhit−/− and Fhit−/− tumors. Statistical tests were two sided and were considered significant at P < 0.05.

### Table 1. Incidence and volume of NNK-induced lung tumors in wild-type and Fhit-deficient mice

<table>
<thead>
<tr>
<th>Genotype (age)</th>
<th>Total tumors</th>
<th>Tumor-bearing mice (%)</th>
<th>Tumors per mouse</th>
<th>Mean volume/± SD</th>
<th>Total volume</th>
<th>Volume/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ (15)</td>
<td>20</td>
<td>16/40 (40)</td>
<td>0.42</td>
<td>0.70 ± 0.66</td>
<td>14</td>
<td>0.35</td>
</tr>
<tr>
<td>+/- (15)</td>
<td>29</td>
<td>14/48 (29)</td>
<td>0.56</td>
<td>1.18 ± 1.62</td>
<td>34</td>
<td>0.71</td>
</tr>
<tr>
<td>-/- (12)</td>
<td>26</td>
<td>2/16 (13)</td>
<td>1.62</td>
<td>1.62 ± 1.60</td>
<td>42</td>
<td>2.62</td>
</tr>
</tbody>
</table>

NOTE: Difference of mean volume was analyzed by Student’s t test: +/+ versus −/− (P = 0.01).

*Age refers to the duration of the experiment in months.

†Volumes expressed in mm³.

### Results

**Lung tumor burden in 4-methylnitrosamino-1-3-pyridyl-1-butanolone-treated Fhit-deficient mice.** Forty Fhit+/+, 48 Fhit+/−, and 16 Fhit−/− B6129 F1 male mice were treated with NNK, as indicated in Materials and Methods, and all mice were alive at the end of the experiment. Whithis, spherical lung nodules were macroscopically detected in lungs of mice of all three genotypes. Table 1 shows the incidence and volume of lung tumors, as assessed 12 to 15 months after the start of the experiment. Although there was a trend toward an increase in the number of tumors per mouse in Fhit−/− mice, we could not determine whether there was a difference in tumor incidence because the homozygous mice were killed 3 months before the other two genotypes. However, lungs of Fhit−/− mice exhibited a significant (P = 0.01) increase in the average tumor volume (1.62 ± 1.60 mm³) compared with the average volume in lungs of wild-type mice (0.70 ± 0.66 mm³). Tumors of Fhit−/− mice showed average volumes intermediate between those of the extreme genotypes. Some tumors from each genotype were histopathologically characterized. Tumors of Fhit−/− mice were all (five of five) well-differentiated bronchiolo-alveolar carcinomas with papillary growth pattern, whereas most tumors of Fhit−/− mice were adenomas and only 17% were carcinomas. No carcinomas were found among tumors of Fhit+/− mice. These results indicate that Fhit deficiency in mouse lung affects tumor size and malignancy.

**Lung cancer in Fhit-Vhl-deficient mice.** A second carcinogen, dimethylnitrosamine, was also used to test the susceptibility of Fhit−/− mice to chemically induced lung tumorigenesis. In a pilot experiment, nine young adult mice were i.p. injected with a single dose of dimethylnitrosamine, four at 2 mg, and five at 6 mg/kg body weight. At ~20 months, mice were sacrificed and tissues analyzed. Solid tumors were not found in the mice treated with the lower dose (one developed a lymphoma). Conversely, in the group treated with the higher dose, two mice (40%) developed lung adenomas. In parallel, we investigated the effect of absence of Fhit, combined with inactivation of one Vhl allele. The tumor phenotype of 31 Fhit−/−/Vhl−/− mice was examined under different experimental conditions. Nine mice were maintained for ~25.5 months without treatment, sacrificed, and lung tissues analyzed for comparison with parental Fhit−/− and Vhl−/− mice of the same age (~24.3 months), as shown in Table 2. Four Fhit−/−/Vhl−/− mice (44%) developed lung adenocarcinomas, whereas none (0 of 10) of the parental Fhit−/− or Vhl−/− mice showed lung neoplasia (P < 0.03), indicating that total absence of Fhit combined with a half normal dose of Vhl protein, is sufficient for lung tumor development in mice. Proliferative...
lesions were noted macroscopically as focal or multifocal, irregular, whitish, firm masses ranging in size from 0.5 to 1.5 mm in diameter; in one case, the lesion was sufficiently large to affect the entire left lung (Fig. 1A-B).

The other 22 Fhit<sup>−/−</sup>/Vhl<sup>+/−</sup> mice were treated with dimethylnitrosamine, as in the previous pilot experiment with Fhit<sup>−/−</sup> mice, with two different doses: 15 mice at 2 mg and 7 mice at 6 mg/kg. The results of this experiment are shown in Table 3. The 15 mice treated with the lower dose were sacrificed at ~19.5 months, lung tissues were analyzed, and were free of lung tumors. Four mice (27%) had liver hemangiomas; the same frequency of hemangiomas found in livers of mice treated with the higher dose (two of seven, 29%). Interestingly, all seven mice treated with 6 mg/kg dimethylnitrosamine and sacrificed at ~19.9 months of age developed lung tumors, five with adenomas, and two with adenocarcinomas. Adenomas were all >1 mm in diameter and, in two of five cases, were multiple (Fig. 1C-D). Lesion multiplicity was not always discernible because some nodules were present only inside lung parenchyma. One of the two adenocarcinomas showed foci of squamous differentiation. A comparison of lung tumor incidences among the different groups, shown in Table 4, permits appreciation of the contribution of the experimental variables. In total, 12 mice were treated with 6 mg/kg dimethylnitrosamine, five Fhit<sup>−/−</sup>/Vhl<sup>+/−</sup>, and seven Fhit<sup>−/−</sup>/Vhl<sup>+/−</sup>. The difference in lung tumor incidence between these two groups (P = 0.04) confirms that after carcinogen exposure, germ line lack of Fhit combined with Vhl half dosage, displays a more pronounced lung tumor phenotype. When tumor incidences of treated and untreated mice are compared, dimethylnitrosamine-treated mice (6 mg/kg), although younger than the untreated cohort, show clear enhancement of lung tumor susceptibility due to carcinogen (P = 0.03). No significant difference due to gender was observed.

To investigate the expression of the remaining Vhl allele in lung tumors, we analyzed, by immunohistochemistry, four adenocarcinomas from Fhit<sup>−/−</sup>/Vhl<sup>+/−</sup> mice and three carcinomas

<table>
<thead>
<tr>
<th>Table 2. Spontaneous phenotypes of aged mice</th>
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<tbody>
<tr>
<td>Mouse</td>
</tr>
<tr>
<td>-------</td>
</tr>
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<td>1</td>
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<td>2</td>
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<td>17</td>
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<tr>
<td>18</td>
</tr>
<tr>
<td>19</td>
</tr>
</tbody>
</table>

Abbreviations: hmg, hemangioma; adca, adenocarcinoma.

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from NNK-treated Fhit-deficient mice (two Fhit−/− and one Fhit+/−) that were wild type for Vhl. As shown in Fig. 2A, all NNK-induced tumors displayed the same intensity of Vhl staining in cancerous and healthy tissues of the same specimen. In tumors from Fhit−/−, Vhl−/− mice, the overall extent of staining with anti-Vhl is fainter in both normal and tumor tissues, as expected due to absence of one Vhl allele. Staining of the tumors for Vhl protein did not definitively show absence of Vhl (Fig. 2B), suggesting that Vhl haploinsufficiency contributes to lung cancer development. Adjacent sections of the same tumors did not show differences of expression of VEGF in tumor and adjacent nontumor tissues after staining with anti-VEGF serum.

Discussion

The FHIT gene was cloned due to its location at human chromosome region 3p14.2 and its interruption by a balanced, familial chromosome translocation that segregated with the occurrence of multifocal, bilateral renal cancers in a three-generation family. After characterization of the locus, gene, and gene product, in many types of cancer, it became clear that expression of Fhit was very frequently lost or reduced in cancers (reviewed in ref. 7), as predicted based on its location at a common fragile region (17). Fhit expression is especially low in human NSCLCs, with ~80% of squamous cell carcinomas and ~50% of adenocarcinomas being negative for Fhit. We have been interested in studying development of kidney, lung, and other cancers in Fhit-deficient mice (9, 10). In fact, the Fhit−/−Vhl−/− cross was initially developed to look for kidney tumors, for which there are not good mouse models, but these mice have not developed kidney cancers using our current protocols.

There are now a number of useful and elegant murine lung cancer models reviewed in ref. (18), including the conditional Ras overexpressing models (3), as well as the p53/Rb conditional knockout mice (4). However, human lung cancers invariably exhibit alterations of genes and loci on chromosome 3p early in tumor development and we are particularly interested in models that mimic the timing and order of genetic/epigenetic alterations occurring in similar human cancers. Two such genes on 3p are FHIT and VHL, for which mouse models are available for tumor susceptibility studies. To our surprise, the Fhit−/−Vhl−/− cross developed spontaneous and induced lung, rather than renal cancers.

Progression in 4-methylnitrosamino-1-3-pyridyl-1-butaneone-induced lung tumors. Wild-type and Fhit-deficient mice, both Fhit−/− and Fhit−/+, did not show significant differences in the incidence of lung tumors following administration of NNK, a tobacco-specific lung carcinogen believed to have a significant role in human lung cancer (19, 20) and known to induce lung tumors in mice (21). This study confirmed our previous findings that spontaneous lung tumors in wild-type and Fhit-deficient mice showed approximately the same incidence (9). However, we observed differential effects on NNK-induced lung tumor size/progression in wild-type and Fhit-deficient mice.

There have been several recent reports concerning the importance of Fhit loss in human cancer progression as a specific clinical feature in at least five types of primary cancer: head and neck and esophageal squamous cell carcinoma, colorectal, breast, and cervical cancers reviewed (7). Additionally, reduced Fhit expression in microinvasive and invasive cervical carcinomas suggested that down-regulation of Fhit is strongly linked to cancer progression and highly aggressive phenotypes (22). Absent or reduced expression of Fhit correlated significantly with diffuse type, poor differentiation, and advanced stage of gastric cancer (23). Some studies have focused specifically on the relationship between loss of Fhit and tumor progression in lung cancer. Association of aberrant Fhit expression with a high rate of tumor cell proliferation was found in lung carcinomas (24) and loss of Fhit was more frequent in invasive lung adenocarcinomas than in earlier stages of the disease (25). Based on the histologic diversity of lung cancers, molecular information has been summarized and stepwise malignant progression models of adenocarcinoma, squamous cell carcinoma, and small cell carcinoma constructed (26). For each model, loss of Fhit expression, together with inactivation of other genes on chromosome 3p, is proposed to play an important role in early stages of cancer development. Thus, the NNK experiment suggests that Fhit-deficient mice may be useful in studying the role of the tobacco smoke carcinogens in lung cancer progression.

Table 3. Phenotypes of mice treated with dimethylnitrosamine (6 mg/kg)

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age (mo)</th>
<th>Genotype</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>20</td>
<td>Fhit−/−</td>
<td>Lung ads</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>Fhit−/−</td>
<td>Lung ads</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>Fhit−/−</td>
<td>Normal</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>Fhit−/−</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>Fhit−/−</td>
<td>Hepatic cyst</td>
</tr>
<tr>
<td>16</td>
<td>19</td>
<td>Fhit−/−Vhl−/−</td>
<td>Lung ads, liver hmg</td>
</tr>
<tr>
<td>17</td>
<td>20</td>
<td>Fhit−/−Vhl−/−</td>
<td>Lung ads, liver hmg</td>
</tr>
<tr>
<td>18</td>
<td>20</td>
<td>Fhit−/−Vhl−/−</td>
<td>Lung ads</td>
</tr>
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<td>19</td>
<td>20</td>
<td>Fhit−/−Vhl−/−</td>
<td>Lung ads</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>Fhit−/−Vhl−/−</td>
<td>Lung ads</td>
</tr>
<tr>
<td>21</td>
<td>20</td>
<td>Fhit−/−Vhl−/−</td>
<td>Lung adca, sq. different.</td>
</tr>
<tr>
<td>22</td>
<td>20</td>
<td>Fhit−/−Vhl−/−</td>
<td>Lung adca, liver cyst</td>
</tr>
</tbody>
</table>

NOTE: ads, adenomas; hmg, hemangioma; adca, adenocarcinoma; sq, squamous.

Table 4. Lung tumor incidences in untreated and dimethylnitrosamine-treated mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (mo ± SD)</th>
<th>Dimethylnitrosamine (mg/kg)</th>
<th>Tumor-bearing mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fhit−/−</td>
<td>20 ± 0.0</td>
<td>6</td>
<td>2/5 (40)</td>
</tr>
<tr>
<td>Fhit−/−Vhl−/−</td>
<td>25.5 ± 6.4</td>
<td>0</td>
<td>4/9 (44)</td>
</tr>
<tr>
<td>Fhit−/−Vhl−/−</td>
<td>19.8 ± 0.4</td>
<td>6</td>
<td>7/7 (100)</td>
</tr>
</tbody>
</table>

NOTE: Tumor incidence was analyzed by two-tailed Fisher’s exact test: Fhit−/− versus treated Fhit−/−Vhl−/− (P = 0.045); untreated versus treated Fhit−/−Vhl−/− (P = 0.034). Significance of age difference was determined by Student’s t-test: untreated versus treated Fhit−/−Vhl−/− (P = 0.034). Previous spontaneous lung tumor incidences for Fhit−/− and Fhit−/− mice were 8.3% and 5.9%, respectively (unpublished results).
Lung tumor induction by NNK showed differences in tumor size and pathologic features in wild-type and Fhit−/− mice, with intermediate tumor sizes occurring in Fhit+/− mice, suggesting that lung tumor susceptibility is exquisitely sensitive to Fhit protein level. This conclusion accords well with earlier observations that most squamous cell carcinomas and about half of adenocarcinomas of the lung are completely negative for Fhit expression, unlike cancers of the breast, kidney, and other organs that frequently show reduced rather than absent Fhit protein. This suggests that even low levels of Fhit, or low activity of the Fhit pathway, may be enough to suppress or prevent lung cancers.

Vhl contribution to lung cancer in Fhit-deficient mice. The incidence of pulmonary tumor lesions observed in Fhit−/− Vhl+/− mice is dependent on the concomitant deficiency of both tumor suppressor genes. Neither Fhit−/− mice (9, 27) nor Vhl−/− mice (13, 14, 28) develop spontaneous lung tumors. Of particular interest in the present study, is the incidence of carcinomas in aging mice. In our control group, none of the 10 mice at 2 years of age, single Vhl+/+ or Fhit−/− developed lung carcinomas. Similar findings were previously described (29). On the other hand, Fhit−/− Vhl+/− mice of matching strain and age showed a carcinoma incidence of 44%, revealing the cooperative action of Fhit and Vhl in murine lung cancer suppression. Interestingly, lungs are among the few tissues that showed enhanced expression of both Fhit (30) and Vhl proteins (31) during mouse embryogenesis. The short arm of chromosome 3 was among the earliest regions reported to show loss of heterozygosity in human cancers (32) and it is now known that at least five regions between 3p12 and 3pter are involved in loss of heterozygosity in up to 100% of lung cancers, depending on the specific region. The VHL gene at 3p25 and the FHIT gene at 3p14.2 are completely inactivated in a large fraction of human cancers through deletion, mutation, epigenetic changes, or a combination of these mechanisms. A prominent function of the Vhl protein is in regulation of proteins involved in angiogenesis,

Figure 2. Detection of Vhl expression by immunohistochemistry. A, NNK treated Fhit−/− lung: nontumor tissue (top), carcinoma is visible (bottom). No difference in staining between the two types of tissues was observed. B, Fhit−/− Vhl+/− lung: intensity of staining in the carcinoma (bottom) and adjacent nontumor tissue (top) is reduced in comparison with NNK tumors, due to the absence of one Vhl allele, but the sensitivity of this immunohistochemical staining does not allow us to conclude definitively that the tumors are Vhl negative.
and Fhit expression can induce an apoptotic pathway; thus, inactivation of these two genes contributes signal pathway changes that are essential to cancer development (33). Because malignancy is a multistep process, it is perhaps not surprising that neither Fhit nor Vhl knockout mice develop lung cancer spontaneously (34). Although in some studies VHL alleles were reportedly lost in the majority of human NSCLCs (12, 35), the gene is not commonly mutated in lung cancers (11), and its contribution to the development of this neoplasia has been controversial. In spite of the immunohistochemical analysis of the tumors of Fhit+/−/Vhl−/− mice, we could not show definitively whether the second Vhl allele is still expressed, suggesting the possibility that loss of one Vhl allele is sufficient, in combination with loss of another 3p suppressor, to promote lung tumorigenesis. This would be an important finding because numerous studies have shown loss of heterozygosity at the VHL locus in human lung cancers, without mutation or inactivation of the second VHL allele. Thus, some investigators have considered that this region must harbor another lung cancer suppressor. If Vhl haploinsufficiency contributes to lung cancer development, the search for another tumor suppressor in 3p25 may be fruitless. Further investigation of the Fhit+/−/Vhl−/− mice may answer this question.

Dimethylhydrazine, a contaminant of some food, air, and smoke (36) is the most potent carcinogen of several common environmental nitrosamines (37). It induces lung tumors in mice (38) and was used in this experiment to verify sensitivity to lung tumor induction in different genotypes. Dimethylhydrazine treatment revealed that Fhit−/−/Vhl−/− mice are more sensitive to chemically induced lung carcinogenesis than Fhit knockout mice. Dimethylhydrazine-treated Fhit+/−/Vhl−/− mice developed both adenomas and carcinomas, whereas aging Fhit+/−/Vhl+/− mice and single Fhit knockout mice developed only carcinomas and adenomas, respectively. The fact that Fhit-deficient mice, in which the Fhit gene product is absent in all cells, do not necessarily develop lung cancers spontaneously, is in accord with numerous observation of loss of Fhit expression in apparently normal lung tissues of smokers (39). Fhit-deficient cells require further genetic or epigenetic alterations for neoplastic progression.

Very recently, Xian et al. (40) have examined mice deficient for one allele of the Dutt1/Robo1 gene, a 3p12 tumor suppressor, for development of spontaneous lung cancers and have observed that 22% of Dutt1+/− mice, at ~20 months of age, developed tumors, 25% of which were invasive lung adenocarcinoma, a significant increase over the 2.8% of wild-type mice that developed small, noninvasive lung tumors. Dutt1 disruption is the first of the human 3p homologous suppressor genes that has been shown to lead to spontaneous mouse lung cancers, but its role in human lung cancer is still under investigation (40). We have now shown that 44% of Fhit-Vhl doubly deficient mice develop spontaneous adenocarcinomas by 2 years of age, suggesting that it will indeed be possible to design animal models of lung cancer that develop through genetic alteration pathways that recapitulate the natural history of human lung cancers. A Fhit-Vhl-Dutt1-deficient cross would be a natural next step in developing mouse models to mimic human lung cancer development, for the study of initiation, progression, prognosis, as well as prevention and therapy.

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References

29. Kryimenova E, Everett JH, Pluta L, Portis M, Gnarra JR, Walker CL. Susceptibility to vascular neoplasms...
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