A Germ-Line p53 Mutation Accelerates Pulmonary Tumorigenesis: p53-independent Efficacy of Chemopreventive Agents Green Tea or Dexamethasone/myo-Inositol and Chemotherapeutic Agents Taxol or Adriamycin

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ABSTRACT

Recent evidence indicates that individuals with a p53 germ-line mutation (Li-Fraumeni syndrome) have a 50% risk of developing lung cancer by age 60. In this study, p53 heterozygous knockout mice and p53 transgenic mice carrying a dominant negative mutation were crossed with the A/J mouse, which is highly susceptible to lung tumor induction, to investigate whether a p53 germ-line mutation is a predisposing gene for carcinogen-induced pulmonary adenomas in mice. The number of lung tumors was not significantly increased in (TSG-p53 × A/J)F1, p53 heterozygous knockout mice as compared with that in (TSG-p53 × A/J)F1 wt mice 16 weeks after exposure to N-nitrosomethylurea (MNNU). In contrast, an average of 22 lung tumors were observed in (UL53-3 × A/J)F1, mice carrying a mutant p53 transgene (135Valp53) compared with an average of 7 lung tumors seen in (UL53-3 × A/J)F1 wt mice after treatment with N-nitrosomethylurea. Similar enhancement of lung tumor multiplicity (~3-fold) was seen when mutant versus wt mice were treated with the tobacco-related carcinogens benzo[a]pyrene or 4-(methylnitrosamino)-(1-3-pyridyl)-1-butaneone. These results suggest that the mutant p53 transgene may have a dominant negative effect on the wt p53. The potential usefulness of this new mouse model in lung cancer chemoprevention and chemotherapy was examined. The chemopreventive efficacy of the green tea or a combination of dietary dexamethasone and myo-inositol and the chemotherapeutic efficacy of Taxol or Adriamycin was examined in wt mice or mice with a mutation in the p53 gene. Mice treated with dexamethasone/myo-inositol and green tea displayed an average of 70 and 50% inhibition of lung tumors, respectively, regardless of p53 status. Similarly, when mice bearing established lung adenomas were treated with Taxol or Adriamycin, a decrease in tumor volume of ~70% was observed independent of p53 mutation status. Thus, the (UL53-3 × A/J)F1 mice treated with dexamethasone/myo-inositol and green tea or the (UL53-3 × A/J)F1 mice treated with Taxol or Adriamycin represent a novel model in which to examine the efficacy of chemopreventive and chemotherapy agents in p53 mutant mice.

INTRODUCTION

There are at least two major findings that spur interest in a mouse model that develops lung cancers with a mutation in p53: (a) p53 mutations are common in human lung cancer, and lung cancer is the most common cause of cancer death in both men and women in the Western World; and (b) LFS, an autosomal dominant disorder, is characterized by germ-line mutations of the p53 gene in approximately 50% of families (1–4). LFS is characterized clinically by frequent occurrence of various cancers including breast cancer, sarcomas, brain tumors, leukemias, adrenocortical tumors, and lung cancer (3–6). One of the most striking cancer types among p53 germ-line mutation carriers is lung cancer (6, 7–10). Recently, Strong et al. found that p53 mutation carriers in LFS families are at a significantly increased risk for lung cancer. The overall risk of lung cancer was approximately 50% in p53 germ-line mutation carriers, implying an extraordinarily high incidence in smokers with the LFS. The p53 nuclear protein consists of at least three domains including: (a) a transactivation domain at the NH2 terminus; (b) a central specific DNA binding domain; and (c) an oligomerization domain at the COOH terminus of the molecule (11, 12). In response to DNA damage, p53 affects transcriptional regulation of gene expression and inhibits tumor cell growth by either inducing G1 arrest or apoptosis (11, 13, 14). Mice, homozygous for the p53-null mutation, are viable and develop a variety of spontaneous malignancies, primarily lymphomas and sarcomas (15–18). A second transgenic mouse containing an Ala-to-Val mutation at codon 135 of the p53 gene developed a high incidence of spontaneous osteosarcoma, lymphoma, and lung adenocarcinoma (19). One goal of the present study was to examine effects of the p53 null mutation and p53 mutant transgene (Val135) on lung tumorigenesis using F1 hybrids derived from crossing p53 null mice (C57BL/6J mice null carrying a p53 null mutation) or UL53-3 mice (FVB/J mice carrying three copies of the p53 transgene) to A/J mice. A/J mice are highly susceptible to both spontaneously occurring and chemically induced lung tumors and routinely develop adenomas with mutations in the K-ras proto-oncogene (20, 21).

p53 mutations are among the most common alterations observed in human cancer, and loss of the p53-dependent cell cycle checkpoints and defects in p53-dependent apoptosis may be a significant impediment to successful cancer therapy (22, 23). However, there is a very low frequency of p53 inactivation in lung tumors from the commonly used A/J mouse lung tumor model (24, 25). Thus, there is an urgent need to develop an in situ mouse model with mutations in both p53 and K-ras that could be used to identify chemopreventive and chemotherapeutic regimens for lung cancer. We have examined the efficacy of two chemopreventive regimens (green tea or combination of dietary dexamethasone and myo-inositol) previously shown to be efficacious in the A/J mouse model (26–31), as well as two chemotherapeutic agents: Taxol and Adriamycin. This study represents the first attempt to systematically validate a p53 transgenic mouse model for lung cancer chemoprevention and chemotherapy.
transgene by PCR: Gsp-1 coincided with the end of intron 4; Gsp-2 matched a 106-bp deletion of exon 5 and a 350-bp deletion of intron 4 (15). We used three genomic transgene was constructed using a targeting vector that contained 3.7 kb of 9 p53 neo TGA-3; and GSP-3 (9 -G TCT CAC GAC CTC CGT CAT GTG CTG -T GGG ACA GCC AAG TCT GTT ATG TGC 3), was developed using primers from the regions of the mouse p53 intron 4 and 2 was prepared in tricarypin. Dexamethasone (>99% pure) and myo-inositol (>99% pure) were purchased from Sigma Chemical Co. Bulk green tea extract powder was 0.1 ml of tricaprylin.

MATERIALS AND METHODS

Reagents. MNU (99% pure), Taxol (>99% pure), Adriamycin (>99% pure), and tricarylpin were obtained from Sigma (St. Louis, MO); NNK (99% pure) was from Chemysm Science Laboratories (Lenexa, KS); and B(α)P (99% pure) was from Aldrich (Milwaukee, Wisconsin). Chemical carcinogens were prepared immediately before use in bioassays: MNU was dissolved in normal saline, NNK was dissolved in warmed PBS, and B(α)P was prepared in tricarypin. Dexamethasone (>99% pure) and myo-inositol (>99% pure) were purchased from Sigma Chemical Co. Bulk green tea extract powder was obtained from the National Cancer Institute.

Animals. p53-deficient mice (TSG-p53) carrying a germ-line null p53 mutation were obtained from GenPharm International Co., Ltd. (Palo Alto, CA). UL53–3 mice, carrying three copies of a transgene containing a 135Val p53 mutation, were obtained from National Institute of Environmental Health Sciences (Research Triangle Park, NC). A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in plastic cages with hardwood bedding and dust covers, in a HEPA-filtered, environmentally controlled room (24 ± 1°C, 12/12 h light/dark cycle). Animals were given Rodent Lab Chow (5001, Purina) and water ad libitum. After a 7-day quarantine, the animals were paired to set up breeding colonies for production of (TSG-p53 × A/J)F1 and (UL53–3 × A/J)F1 mice. For the chemopreventive and chemotherapeutic studies, mice were fed powdered AIN-76A Purified Diet (100000, Dyets Inc., Bethlehem, PA). Body weights were monitored monthly for the duration of the studies.

p53 Genotype. Tail clippings from each (TSG-p53 × A/J)F1 and (UL53–3 × A/J)F1 mouse were homogenized and incubated overnight at 37°C in lysis solution (promaze 0.4 mg/ml, 10% SDS (w/v), 10 mM Tris, 400 mM NaCl, and 2 mM EDTA) followed by phenol-chloroform extraction and precipitation with ice-cold alcohol. For genotyping (TSG-p53 × A/J)F1, mouse a new PCR method was developed using primers from the regions of the mouse p53 intron 4 and exon 5 in which the TSG-p53 and a Pol II-neo expression cassette (15). This screening was repeated at least once for confirmation. The “knockout” p53 transgene was constructed using a targeting vector that contained 3.7 kb of genomic p53 and a Pol II-neo expression cassette (15). By homologous recombination, the targeting construct inserted itself into the p53 gene, causing a 106-bp deletion of exon 5 and a 350-bp deletion of intron 4 (15). We used three primers, GSP-1, GSP-2, and GSP-3, for screening of the knockout transgene by PCR: Gsp-1 coincided with the end of intron 4; Gsp-2 matched the sequence for the middle of exon 5; and Gsp-3 was specific for the neo cassette. As shown in Fig. 1A, a fragment of 960 bp was amplified in the wt p53 (wt/twt) with primers GSP-1 and GSP-2 (Lanes 1 and 8); however, when the neo gene construct is present, a diagnostic 120-bp fragment is generated using primers GSP-1 and GSP-3 (Lanes 5). DNA with the wt allele and null transgenic allele showed 960-bp and 120-bp bands (Lanes 2, 4, 6–7, and 9–10). This screening was repeated at least once for confirmation.

Using the UL53–3 mice were developed by microinjection of FVB/J mouse oocytes with a BALB/c mouse genomic clone of the p53 gene containing a point mutation at codon 135 (Ala→Val) in exon 5 of the p53 gene. The p53 transgene used in the generation of the UL53–3 mice was the same as that used in a previous study (19). The mutation, a C→T transition, created a RFLP with a new Hph restriction enzyme cleavage site (recognition site: GTTGA). This mutation was used to genotype (UL53–3 × A/J)F1 mice using the PCR–RFLP method. As shown in Fig. 1B, a single 190-bp band was amplified for wt p53 mice (Lanes 4–7 and 9), whereas three bands (190-bp, 150-bp, and 40-bp) were obtained in heterozygous p53 transgenic mice (Lanes 1–3 and 8). Specifically, PCR primers were designed from the regions of mouse p53 exon 5 that contained the Ala→Val mutation. The primer sequences were as follows: 5′-TAC TCT CCT CCC CTC AAT AAG-3′; and 5′-CTG TTC TGG TTC CCA ATA GAG-3′. PCR conditions were the same as those described above. These PCR primers generated a 190-bp-amplified exon 5 fragment from both the wt p53 allele and the mut transgene allele. After amplification, the fragment was incubated with the restriction endonuclease HphI, which cleaves once within the amplified mut transgene and none within the wt allele. The cleaved fragments were then subjected to electrophoresis on an 8% polyacrylamide gel along with a DNA size marker and visualized by UV light after staining with ethidium bromide. This procedure was also repeated at least once for each mouse for confirmation.

Lung Tumorigenesis Studies. Six-week-old (TSG-p53 × A/J)F1 and (UL53–3 × A/J)F1 hybrid mice were randomized into eight groups according to the p53 genotypes and treatments in the lung tumor bioassay using MNU. As seen in Table 1, mice in groups 1, 2, 5, and 6 were given a single i.p. injection of 0.1 ml normal saline as vehicle controls. Mice in groups 3, 4, 7, and 8 and were given a single injection of MNU (50 mg/kg body weight) in 0.1 ml normal saline. Sixteen weeks after exposure to MNU, animals from all of the eight groups were killed by CO2 asphyxiation. The lungs were fixed in Tellyeninsky’s solution overnight, followed by 70% ethanol treatment. The number of tumors for each lung was counted by two independent investigators (Z.Z., Q.L.) using a dissecting microscope.

Six-week-old (UL53–3 × A/J)F1 mice were randomized into six groups for

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
<th>No. of mice</th>
<th>Treatment</th>
<th>Incidence</th>
<th>Tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p53&lt;sup&gt;wt/WT&lt;/sup&gt;</td>
<td>20</td>
<td>Vehicle</td>
<td>0/20 (0%)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>p53&lt;sup&gt;Val135/WT&lt;/sup&gt;</td>
<td>20</td>
<td>Vehicle</td>
<td>1/20 (10%)</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>p53&lt;sup&gt;Val135/wt&lt;/sup&gt;</td>
<td>21</td>
<td>NNK</td>
<td>16/21 (76%)</td>
<td>1.1 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>p53&lt;sup&gt;Val135/wt&lt;/sup&gt;</td>
<td>20</td>
<td>NNK</td>
<td>20/20 (100%)</td>
<td>3.2 ± 1.1*</td>
</tr>
<tr>
<td>5</td>
<td>p53&lt;sup&gt;Val135/wt&lt;/sup&gt;</td>
<td>20</td>
<td>B(α)P</td>
<td>20/20 (100%)</td>
<td>3.1 ± 1.8</td>
</tr>
<tr>
<td>6</td>
<td>p53&lt;sup&gt;Val135/wt&lt;/sup&gt;</td>
<td>19</td>
<td>B(α)P</td>
<td>19/19 (100%)</td>
<td>11.3 ± 5.4*</td>
</tr>
</tbody>
</table>

a At 6 weeks of age, mice (equal numbers of males and females) in groups 1 and 2 were given vehicle. Mice in groups 3 and 4 were given two i.p. injections of MNU (100 mg/kg body weight) in 0.1 ml of PBS 1 week apart, and mice in groups 5 and 6 were given a single i.p. injection of B(α)P (100 mg/kg body weight) in 0.1 ml of tricarypin.

b P < 0.0001, tumor multiplicity was significantly different from that of MNU-treated p53<sup>wt/WT</sup> group.

c P < 0.0001, tumor multiplicity was significantly different from that of B(α)P-treated p53<sup>Val135/WT</sup> group.
the lung tumor bioassays with NNK and B[a]P. As seen in Table 2, mice in groups 1 and 2 were given vehicle (50% of the mice received a single i.p. injection of 0.1 ml of tricaprylin, and the remainder were given 2 i.p. injections of 0.1 ml of PBS 1 week apart). Mice in groups 3 and 4 were given two injections of NNK (100 mg/kg body weight) in 0.1 ml of PBS 1 week apart, and mice in groups 5 and 6 were given a single i.p. injection of B[a]P (100 mg/kg body weight) in 0.1 ml of tricaprylin. All of the animals were observed daily for clinical signs of ill health and weighed individually twice a month for the duration of the study. Eighteen weeks after exposure to carcinogen, all of the animals were killed by CO₂ asphyxiation. The lung tumors were counted after the lungs were fixed in Tellyeninsky’s solution. All of the lung tumors were diagnosed as lung adenomas.

**TUNEL Assay.** The hallmark 3’ OH DNA groups were labeled with TdT followed by a direct immunoperoxidase labeling of digoxigenin-labeled genomic DNA using the Apoptag Plus In Situ Apoptosis detection kit (Oncor, Inc., Tucson, AZ). The manufacturer’s protocol was followed with the exception of the working concentration of TdT, which was optimized to a 1:10 dilution. Among controls, negative sections were not treated with TdT; positive sections included weaning-stage rat mammary gland tissue and also normal mouse lung tissue preincubated with 0.5 μg/ml DNase I for 10 min at 25°C, to induce 3’ OH strand breaks detected by the TUNEL method. Positive nuclei were scored both on the basis of labeling with DAB and morphological hallmarks of apoptosis including death of single cells, evidence of membrane blebbing, uniformly dense chromatin masses, cell shrinkage, halo effect around the nucleus, and nuclear versus cytoplasmatic staining at ×4000. The level of apoptosis was determined by counting 10 randomly chosen fields per section and determining the percentage of DAB-positive cells per 100 cells at ×400. The counts were averaged to obtain the AI.

**Immunohistochemistry.** PCNA was detected using a modified indirect immunohistochemistry assay to detect the mouse proteins using mouse monoclonal antibodies. Paraffin-embedded 5-μm sections were deparaffinized and rehydrated followed by blocking endogenous peroxides (3% H₂O₂ in PBS). Before incubation with primary monoclonal antibodies, the sections were preincubated with a mouse-mouse kit (developed by Novartis) per manufacturer’s instructions. PCNA-positive staining was determined by counting 10 randomly chosen fields per section, determining the percentage of DAB-positive cells per 100 cells at ×400.

**Chemoprevention Studies with myo-Inositol and Dexamethasone.** Six-week-old (UL53–3 × A/J)F₁ hybrid mice were randomized into four groups, two each $p^{53 \text{wt/wt}}$, and $p^{53 \text{val/val}}$, of approximately equal numbers of males and females. Control groups 1 and 2 were fed AIN-76A purified diet. Test groups 3 and 4 were given a diet composed of AIN-76A with dexamethasone (0.5 mg/kg diet) and myo-inositol (1%) as described by Wattenberg et al. (26). All of the food and water were available ad libitum. The test diet regimen began 2 weeks before the administration of carcinogen and continued for the duration of the experiment. Mice received two doses of NNK 1 week apart at 100 mg/kg i.p., at 8 and 9 weeks of age. The mice were killed 20 weeks after exposure to NNK by CO₂ asphyxiation. The lungs were fixed in 10% buffered formalin overnight, followed by 10% ethanol. The lung tumors were counted by two independent investigators (Z.Z., Q.L.) using a dissecting microscope prior to paraffin embedding.

**Chemoprevention Studies with Green Tea.** Six-week-old (UL53–3 × A/J)F₁ hybrid mice were randomized into four groups, two each $p^{53 \text{wt/wt}}$ and $p^{53 \text{val/val}}$, of males and females. All of the mice were given two i.p. injections of NNK (100 mg/kg) 1 week apart. Beginning 1 week after the final injection of carcinogen, mice in groups 3 and 4 were given a solution of 0.6% green tea as their sole source of drinking fluid until the end of the experiment as described by Xu et al. (27). Groups 1 and 2 received deionized water. All of the animals were maintained on AIN-76A purified diet. Fluids and food were available ad libitum. The experiment was terminated 20 weeks after exposure to NNK, and the lungs were harvested as stated previously.

**Chemotherapy Studies.** Six-week-old (UL53–3 × A/J)F₁ hybrid mice were randomized into six groups: (Control, groups 1 and 2; Taxol treatment, groups 3 and 4; Adriamycin treatment, groups 5 and 6). All of the animals were given two i.p. doses of NNK at 100 mg/kg 1 week apart. Sixteen weeks after exposure to NNK, the mice in groups 3 and 4 were treated with Taxol, suspended in ethanol/Cremophor EL (1:1 v/v), and further diluted in PBS to deliver 20 mg/kg i.p., in 0.2 ml twice a week for 3 weeks. The mice in groups 5 and 6 were treated with Adriamycin (5 mg/kg body weight) i.p. in 0.2 ml twice a week for 3 weeks. At the end of the test period, the mice were killed by CO₂ asphyxiation, and the lungs were harvested and fixed as described above. The tumors were counted and measured under a dissecting microscope by two independent investigators (Z.Z., Q.L.). Tumor volume ($V$) was calculated by $V = (4/3) \pi r^3$, where $r$ = radius of the lung tumor.

**Statistical Analysis.** One-way ANOVA was used to determine the difference in the number of pulmonary adenomas per mouse between control and treated groups. Two-way ANOVA was used to determine the difference in both the number and the size of lung tumors between control and treated groups.

**RESULTS**

**Effect of Loss of an Allele of the $p^{53}$ Gene on MNU-induced Mouse Lung Carcinogenesis.** A decrease in dosage of the $p^{53}$ gene has been shown to promote tumorigenesis at many sites in $p^{53}$ heterozygous mice (17, 32). To determine whether $p^{53}$ heterozygous mice with one defective allele (knockout) and one normal allele are more susceptible to lung tumorigenesis, two groups of (TSG-p53 × A/J)F₁ mice and two groups of (TSG-p53 × A/J)F₁ $p^{53}$ heterozygous mice were used in a bioassay with MNU. The (TSG-p53 × A/J)F₁ mice were genotyped for the presence of the $p^{53}$ knockout allele using the PCR method as described in “Materials and Methods” (Fig. 1A). A 100%
incidence of lung tumors was observed in both groups of treated mice. As shown in Table 1, treatment of (TSG-p53<sup>3A/J</sup>)F<sub>1</sub>p53 heterozygous mice with MNU induced an average of 7.2 tumors/mouse (n = 25), and treatment of (TSG-p53<sup>3A/J</sup>)F<sub>1</sub>wt mice induced an average of 6.7 tumors/mouse (n = 33). The lung tumors were histologically confirmed as adenomas by light microscopy of H&E-stained sections. No significant difference in tumor multiplicity was observed between (TSG-p53<sup>3A/J</sup>)F<sub>1</sub>wt mice (n = 26) and (TSG-p53<sup>3A/J</sup>)F<sub>1</sub>p53 heterozygous mice (n = 17) in the vehicle controls. These results indicate that a decrease in the wt p53 gene dosage does not enhance MNU-induced lung carcinogenesis in (TSG-p53<sup>3A/J</sup>)F<sub>1</sub> mice.

Effect of a Germ-Line Mutation in the p53 Gene on Carcinogen-induced Mouse Lung Tumorigenesis. To determine whether p53 transgenic mice are more susceptible to lung tumorigenesis, wt and mut (UL53–3<sup>3A/J</sup>)F<sub>1</sub> mice were used in a bioassay with MNU. The (UL53–3<sup>3A/J</sup>)F<sub>1</sub> mice were genotyped for the presence of the p53 mut transgene using the PCR-RFLP method described in “Materials and Methods” (Fig. 1B). As shown in Table 1, (UL53–3<sup>3A/J</sup>)F<sub>1</sub> mice carrying a mut p53 transgene (Val 135) developed a higher number of lung tumors after treatment with MNU than (UL53–3<sup>3A/J</sup>)F<sub>1</sub>p53 wt mice [22.0 ± 5.7 tumors/mouse (n = 14) compared with 7.3 ± 3.5 tumors/mouse (n = 18); P < 0.0001; Table 1]. The

Table 3 Chemopreventive effects of myo-inositol/dexamethasone and green tea on NNK-induced pulmonary carcinogenesis in p53 transgenic mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Incidence</th>
<th>Tumor multiplicity</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p53&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>NNK/Vehicle</td>
<td>19/19 (100%)</td>
<td>5.7 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>p53Val135&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>NNK/Vehicle</td>
<td>19/19 (100%)</td>
<td>16.3 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>p53&lt;sup&gt;Val135&lt;/sup&gt;</td>
<td>NNK/myo + Dex&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>16/18 (89%)</td>
<td>1.7 ± 1.4</td>
<td>70.3%</td>
</tr>
<tr>
<td>4</td>
<td>p53Val135&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>NNK/myo + Dex</td>
<td>18/19 (95%)</td>
<td>4.3 ± 1.8</td>
<td>73.6%</td>
</tr>
<tr>
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<td>20/20 (100%)</td>
<td>5.1 ± 2.8</td>
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<td>6</td>
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<td>20/20 (100%)</td>
<td>14.4 ± 5.3</td>
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<tr>
<td>7</td>
<td>p53&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>NNK/tea&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19/19 (100%)</td>
<td>2.4 ± 2.3</td>
<td>52.9%</td>
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<tr>
<td>8</td>
<td>p53Val135&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>NNK/tea</td>
<td>19/19 (100%)</td>
<td>7.4 ± 3.0</td>
<td>48.7%</td>
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<sup>a</sup> myo, myo-inositol; Dex, dexamethasone.

<sup>b</sup> Six-week-old animals (equal numbers of males and females) were given two i.p. injections of NNK (100 mg/kg body weight), Dex, (0.5 mg/kg food) and myo (1% in the diet) were dietary additions starting 2 weeks prior to the first dose of NNK.

<sup>c</sup> P < 0.0001, significant inhibition of tumor multiplicity compared with corresponding control groups.

<sup>d</sup> At 6 weeks of age, all of the animals (equal numbers of males and females) were given two i.p. injections of NNK (100 mg/kg body weight) 1 week apart. Green tea (0.6%) administered as the sole source of drinking fluid from 1 week after the initiation period (second dose NNK) and full-strength solutions were used thereafter.
incidence of lung tumors in both groups of treated mice was 100%. In the vehicle control groups, a low incidence of lung tumors was observed, and there was no difference in tumor multiplicity between p53\textsuperscript{wt/wt} mice and p53\textsuperscript{val/val} mice (groups 1 and 2).

We also evaluated the effect of the germ-line p53 mutation (Val\textsubscript{135}) on lung tumorigenesis induced by tobacco-associated carcinogens NNK and B[a]P. (UL53–3 × A/J)F\textsubscript{1} mice were treated with B[a]P and NNK using a protocol similar to that described for MNU. As seen in Table 2, treatment of (UL53–3 × A/J)F\textsubscript{1} wt mice with NNK produced an incidence of 76% and multiplicity of 1.1 lung tumors/lung, whereas treatment of (UL53–3 × A/J)F\textsubscript{1} p53 mut mice produced a 100% incidence and multiplicity of 3.2 (Table 2). In the B[a]P-treated group, 100% lung tumor incidence was observed in all of the mice. However, the lung tumor multiplicity in (UL53–3 × A/J)F\textsubscript{1} p53-mut mice (11.3 tumors/mouse; n = 19) was 3.6 times higher than that in (UL53–3 × A/J)F\textsubscript{1} wt mice (3.1 tumors/mouse; n = 20; P < 0.0001).

The possibility that increased mouse lung tumor susceptibility to chemical carcinogens may result from the reduction of apoptosis and/or increased cell proliferation by the p53 mut was investigated. The levels of apoptotic activity in NNK-induced mouse lung tumors from (UL53–3 × A/J)F\textsubscript{1} p53 wt and mut mice were assayed in situ using the ApopTag plus In Situ Apoptosis Detection Kit (Oncor, Inc.). The percentage of apoptotic cells was quantified by counting 10 randomly chosen fields at ×400 to obtain the AI as described in “Materials and Methods.” As shown in Fig. 2, a significant decrease (≈3.0-fold) in the levels of apoptosis was observed in lung tumors from NNK-treated (UL53–3 × A/J)F\textsubscript{1} p53-mut mice (0.36 ± 0.17) as compared with those seen in NNK-treated (UL53–3 × A/J)F\textsubscript{1} wt mice (0.99 ± 0.12; P < 0.05). In addition, cell proliferation rate in NNK-induced lung tumors was determined using antibodies to the cycling-specific protein PCNA (Fig. 2). PCNA indices increased significantly in induced lung tumors following exposure to chemical carcinogens in both p53\textsuperscript{wt/wt} and p53\textsuperscript{val/val} mice of more than 70% (P < 0.0001) compared with those seen in NNK-treated (UL53–3 × A/J)F\textsubscript{1} p53-mut mice (Fig. 2), as compared with those of p53\textsuperscript{mut/mut} mice (3.74 ± 1.1; P < 0.05). These data indicate that the level of apoptosis was significantly reduced, and cell proliferation rate was significantly increased in lung tumors in the presence of p53 mut (Val\textsubscript{135}) gene. The results suggest that the observed increase in the number of the lung tumors following exposure to chemical carcinogens in p53 mut mice may be due in part both to the reduction of p53-dependent apoptosis and the increased rate of cell proliferation.

Chemopreventive Efficacy of Dexamethasone/\textit{myo}-inositol and Green Tea in p53 Transgenic Mice. The incidence and multiplicity of lung tumors in NNK-treated p53\textsuperscript{wt/wt} and p53\textsuperscript{val/val} mice was 100% (with an average of 5.7 tumors/mouse) and 100% (with an average of 16 tumors/mouse), respectively (Table 3). This demonstrates the reproducibility of the enhancement associated with having a mut p53 genotype. As shown in Table 3, treating mice with a combination of dexamethasone and \textit{myo}-inositol, beginning 7 days before NNK administration and continually thereafter, strikingly reduced tumor multiplicity in both the p53\textsuperscript{wt/wt} group (1.7 ± 1.4 tumors per mouse) and the p53\textsuperscript{val/val} group (4.3 ± 1.8 tumors/mouse). This represents a significant inhibition of lung tumor multiplicity in both p53\textsuperscript{wt/wt} and p53\textsuperscript{val/val} mice of more than 70% (P < 0.0001). The administration of green tea as the sole drinking source beginning 1 week after NNK administration significantly reduced tumor multiplicity in both p53\textsuperscript{wt/wt} and p53\textsuperscript{val/val} mice; however, there was no change in incidence compared with control (100%). Tumor multiplicity for the p53\textsuperscript{wt/wt} mice treated with NNK was 5.1 and decreased to 2.4 in mice treated with green tea. Tumor multiplicity for p53\textsuperscript{val/val} mice treated with NNK was 14.4 and decreased to 7.4 with green tea treatment. These data represent a significant reduction in tumor multiplicity in p53\textsuperscript{wt/wt} (52.9%) and in p53\textsuperscript{val/val} (48.7%; P < 0.0001; Table 3).

Although the combination of dexamethasone and \textit{myo}-inositol was given at a dose that was used in a previously published paper (26), a 10% decrease of final body weight was observed. Near the end of the experiment (weeks 19–22), several mice were found dead or moribund. Although we noted a difference in the final body weight (less than 10%) of mice that were treated with green tea, food intake was normal and no other ill effects were noted, consistent with the previously published observation (27).

Chemotherapeutic Efficacy of Taxol and Adriamycin in p53 Transgenic Mice. For the therapy experiments, mice were treated with the indicated therapeutic agents beginning 16 weeks after the time they were given NNK. In our previous studies, by 16 weeks, most mice exhibited multiple lung adenomas (24). In the present study, we evaluated the size and number of tumors using the total tumor volume [Volume = (4/3)\\pi r^3] to assess the efficacy of treatment. As shown in Table 4, Taxol treatment in the p53\textsuperscript{wt/wt} group significantly reduced tumor volume by 61.4% (P < 0.0001) of control values. In p53\textsuperscript{val/val} mice, tumor volume was reduced by 77.3% (P < 0.0001) as compared with control values. Adriamycin treatment also caused a significant reduction in final tumor volume—66.7% in p53\textsuperscript{wt/wt} mice and 72.5% in p53\textsuperscript{val/val} mice. These results indicate that Taxol and Adriamycin are effective chemotherapeutic drugs in mouse lung tumor bioassays, with a significant decrease in tumor volume in both p53\textsuperscript{wt/wt} and p53\textsuperscript{val/val} mice. Furthermore, both Taxol and Adriamycin show greater (but not statistically significant) efficacy in reducing tumor volume in p53\textsuperscript{wt/wt} mice as compared with p53\textsuperscript{val/val} mice.

**DISCUSSION**

A high percentage of individuals with LFS who smoke develop lung cancer (Refs. 6, 7–10; Strong et al.).

\[6 \text{ L. C. Strong, personal communication.} \]
to analyze the effects of a germ-line p53 defect on lung tumor susceptibility and development. We observed that (UL53-3 × A/J)F1 p53 mut mice carrying a p53 transgene (Val135) exhibit an increased susceptibility to the chemical induction of lung cancer by various carcinogens. Because these (UL53-3 × A/J)F1 p53-mut mice retain both copies of the normal p53 alleles (data not shown), it implies that the introduction of a p53 transgene expressing the mut p53 protein (Val135) inactivates endogenous wt p53 (34).

We found that (TSG-p53 × A/J)F1 p53 mice that are heterozygous for a null mutation were not more susceptible to chemically induced lung adenomas despite the important role p53 plays in cell cycle checkpoint control and apoptosis. Our data are consistent with results from Kemp et al. (35), who showed that neither heterozygous nor homozygous knockout of the p53 gene affected the development of TPA-promoted mouse skin papillomas. However, an increased rate of malignant progression was observed in both p53 heterozygous and p53 homozygous knockout mice (35). The present study was terminated 4–5 months after administration of carcinogen, which was too early to observe any malignant conversion of lung adenomas into lung adenocarcinomas. Interestingly, these heterozygous p53 null (knock-out) mice are more susceptible to the chemical induction of cancer in certain other organ sites, for example, the bladder (32).

Our finding that (UL53-3 × A/J)F1 p53 mut mice (Val135) are highly susceptible to the chemical induction of mouse lung tumors should provide an important mouse model to study the role of p53 in lung tumorigenesis. First, the model is directly related to patients with LFS who develop lung cancer at a remarkably high rate. Not unexpectedly, these patients are typically smokers. For example, a 57-year-old female carrier who was a heavy smoker developed multiple synchronous lung cancers (9). In fact, lung cancer was found to be the most frequently observed cancer type in adult male p53 mutation carriers with a 50% risk of developing lung cancer by age 60.7 Whereas <10% of smokers develop lung cancer, almost 50% of males with LFS develop lung cancer. If individuals with LFS have the average incidence of smokers, this implies a remarkably high penetrance (>80%). This agrees with our results with p53 transgenic mice, which indicated the contribution of p53 mutations to the initial stages of the carcinogenic process. Furthermore, there is a 4-fold increase in lung cancer incidence among carriers with p53 mutations who are smokers as compared with those who are non-smokers,8 which suggests a profound interaction between tobacco smoke and p53 mutations. This result would seem to parallel our finding of increased susceptibility to two major tobacco-related carcinogens—NNK and B[a]P. In addition to being a highly relevant model for LFS, our model generates tumors with multiple genetic lesions that are frequently seen in human lung cancers also (21). Most of the lung adenomas (>80%) from A/J mice have activating mutations in the K-ras proto-oncogene, and more than 50% of the lung adenocarcinomas display loss of heterozygosity of the p16INK4a gene (22). More recent studies have not yielded such clear-cut results. Thus, a recent in vivo study examining the long-term effects of anthracyclines on tumor growth and proliferation has not shown a clear association with p53 status (39). Our results suggest that the status of p53 in tumor cells may not be by itself be the primary determinant of response to anticancer treatment for many agents. The use of this model, with alterations in both the K-ras and p53 genes, for assessing cancer prevention and, in particular, cancer chemotherapeutic agents should be further explored.

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