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Mice with Alterations in Both p53 and Ink4a/Arf Display a Striking Increase in Lung Tumor Multiplicity and Progression: Differential Chemopreventive Effect of Budesonide in Wild-type and Mutant A/J Mice[1]

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ABSTRACT

p53 transgenic mice carrying a dominant negative mutation were crossed with Ink4A/Arf heterozygous-deficient mice to investigate whether there is a synergy between these two germ-line mutations in promoting carcinogen-induced lung tumor progression in mice. Mice with a p53 dominant negative mutation and Ink4A/Arf heterozygous deficiency exhibited >20-fold increase in tumor volume compared with ~4-fold increase in Ink4A/Arf heterozygous-deficient mice and a 9-fold increase in mice with only the p53 dominant negative mutation. The effect of Ink4A/Arf heterozygous deficiency on lung tumor progression occurred late in the carcinogenesis process (>30 weeks after carcinogen treatment). In addition, most of the lung tumors (~80%) from mice with a p53 mutation and deletion of Ink4A/Arf were lung adenocarcinomas. In contrast, lung adenocarcinomas were seen in <10% of the lung tumors from the wild-type mice and ~50% of the lung tumors from Ink4a/Arf heterozygous-deficient or p53 mutant mice. These results indicate a significant synergistic interaction between the presence of a mutant p53 transgene and the Ink4A/Arf deletion during lung tumor progression ($P < 0.01$). The usefulness of this new mouse model in lung cancer chemoprevention was examined. The chemopreventive efficacy of budesonide was examined in wild-type mice, mice with Ink4A/Arf heterozygous deficiency, mice with a mutation in the *p53* gene, or mice with both a mutation in the *p53* gene and deletion in the Ink4A/Arf locus. Mice treated with budesonide displayed an average of 90% inhibition of lung tumor progression in a standard 18-week chemoprevention assay, regardless of p53 and/or Ink4A/Arf status. However, the efficacy of budesonide against lung tumor progression decreased from 94 to 77% ($P = 0.07$) in mice with alterations in both p53 and Ink4A/Arf in a 40-week chemoprevention assay. Similarly, when mice bearing established lung adenomas were treated with budesonide, genotype-dependent differential effects of budesonide in wild-type and mutant mice were clearly revealed with a 82, 64, 45, and 33% decrease in tumor volume in wild-type mice, p53$^{+/+}$Ink4a/Arf$^{+/-}$ mice, p53$^{+/-}$Ink4a/Arf$^{+/+}$ mice, and p53$^{+/-}$Ink4a/Arf$^{+/-}$, respectively. Thus, mutant mice with alterations in p53 and/or Ink4A/Arf exhibited a significant resistance to chemoprevention by budesonide. Because p53 and Ink4a/Arf mutations are the most prevalent mutations in human lung cancers, the effectiveness of chemopreventive agents on the mutant A/J mice containing alterations with p53 and Ink4a/Arf is the best preclinical estimate of their efficacy in humans. Thus, the mutant A/J mouse model should prove useful for chemoprevention studies.

INTRODUCTION

Several genetic changes, including genetic alterations in Kras2, p53, and Ink4a/Arf, are commonly found in human lung cancers, especially in human lung adenocarcinomas (1, 2). Activating point mutations in the *Kras2* gene have been detected in 30–50% of lung adenocarcinomas (1, 2). Activated Kras2 has also been detected in spontaneously occurring and chemical-induced mouse lung tumors (2, 3). The frequency of occurrence of activated *Kras2* genes depends on the relative susceptibility of the mouse strain and carcinogen treatment protocol used to induce tumors (4–8). Recently, we performed a lung tumor bioassay in heterozygous Kras2-deficient mice to evaluate the effect of the presence of a wild-type *Kras2* allele on lung tumorigenesis (9). Mice with a heterozygous K-*ras* deficiency had an increased susceptibility to the chemical induction of lung tumors when compared with wild-type mice, suggesting that loss of wild-type *Kras2* allele promotes activating Kras2 mutation-driven lung carcinogenesis (9).

p53 mutations are common in human lung cancer (1, 2). p53 mutation carriers in LFS[4] families are at a significantly increased risk for several cancer types, including lung cancer (10).[5] Two primary mouse models for human LFS have been developed. The first involves knockout of one or both copies of the *p53* gene (11–14), and these mice are viable and develop a variety of spontaneous malignancies, primarily lymphomas, and sarcomas. The second has involved transgenic expression of a dominant negative mutant p53 in animals still retaining copies of the wt of p53 (15). These mice developed a high incidence of spontaneous osteosarcoma, lymphoma, and lung adenocarcinoma (15). We have evaluated previously the effect of the *p53* gene on mouse lung carcinogenesis using both p53 heterozygous knockout mice and p53 transgenic mice carrying a dominant negative mutation by crossing them with lung tumor susceptible A/J mice (16). Although there is no change in lung tumorigenesis in p53 heterozygous knockout mice comparing with wt mice, a 3-fold increase in lung tumor multiplicity was observed, suggesting that the mutant p53 transgene may have a dominant negative effect on the wt p53 (16).

The Ink4a/Arf locus encodes two functionally distinct tumor suppressors, p16^{INK4a} and ARF (17, 18). The *p16^{INK4a}* gene codes for an inhibitor of the cyclin D–cyclin-dependent kinase complex and thereby affects the Rb/E2F pathway and cell cycle progression (19). Several studies indicated that the *p16^{INK4a}* and *Rb* genes are reciprocally inactivated in lung cancer cells, although the *RB* and *p16^{INK4a}* genes are preferentially altered in small cell lung carcinoma and NSCLC, respectively (20–22). Hypermethylation of the *p16^{INK4a}* gene is frequently observed in NSCLC, and ~50% of losses of p16^{INK4a} expression in NSCLC can be caused by hypermethylation of the 5′ regulatory region in the *p16^{INK4a}* gene (22–24). Because of genetic alterations, the signaling pathway via RB and p16^{INK4a} is estimated as being disturbed in most lung cancers. ARF is encoded by the *Cdkn2a* gene in which an alternative first exon is spliced into exon 2 in a reading frame different from that of p16^{INK4a} (21, 22). p16^{INK4a} and ARF are immunologically and functionally distinct proteins (21, 22). ARF is capable of inhibiting cell proliferation by blocking the ubiquitin E3 ligase activity of MDM2 (25) and by sequestering

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[4] The abbreviations used are: LFS, Li-Fraumeni syndrome; wt, wild type; BP, benzo(*a*)pyrene; NSCLC, non-small cell lung carcinoma; ARF, ADP ribosylation factor; Rb, retinoblastoma.

[5] L. C. Strong, personal communication.

MDM2 into the nucleolus (26, 27). ARF can be induced by oncogenic signals and acts upstream of p53 to negatively regulate the effects of specific stresses, including oncogenic signaling, DNA damage, and microtubule disruption (28, 29). Decreased expression of ARF has been found in 60% of lung cancers (30). Recently, we reported that mice with an Ink4a/Arf heterozygous deficiency exhibited an increase in lung tumor progression (31). Thus, the Ink4a/Arf locus encodes two proteins that regulate two very important tumor suppressor pathways for lung cancer, RB and p53.

The first goal of the present study was to examine the effect of alterations of both p53 and Ink4a/Arf on lung tumorigenesis using A/J mice carrying germ-line alterations in both genes ($p53^{val135/wt}$ and $Ink4a/Arf^{+/-}$). This was accomplished by backcrossing UL53-3 mice (FVB/J mice carrying three copies of the p53 transgene) to A/J mice for 10 generations and 129-B6 mixed mice (Ink4a/Arf^{KO}) to A/J mice for 6 generations, respectively. A/J mice are highly susceptible to both spontaneously occurring and chemically induced lung tumors and routinely develop adenomas and adenocarcinomas with mutations in the K-*ras* proto-oncogene (3, 32). We saw a significant functional synergy among the presence of genetic alterations in Kras2, p53, and Ink4a/Arf in promoting lung carcinogenesis. Moreover, this study led to the development of an *in situ* mouse model with mutations in Kras2, p53, and/or Ink4a/Arf that could be used to identify chemopreventive regimens for lung cancer. The second goal of this study was to determine the efficacy in these mice of a potent chemopreventive agent, budesonide, which has been shown previously to be highly efficacious in the A/J mouse model (2, 3). This was used to systematically validate a p53-Ink4a/Arf transgenic mouse model for lung cancer chemoprevention. Budesonide is a synthetic glucocorticoid and has exhibited a strong preventive activity in BP-induced pulmonary adenoma formation in the female A/J mouse model. This inhibitory effect was achieved with budesonide administered either by diet and/or aerosol even at low levels (33–36). Budesonide was one of a few compounds with lung tumor inhibitory effects even when administered after carcinogen initiation (34). In addition, budesonide is currently undergoing human trails. Accordingly, budesonide is selected as a model compound for the present study.

MATERIALS AND METHODS

Reagents. BP (99% pure), budesonide (>99% pure), and tricaprylin were obtained from Sigma Chemical Co. (St. Louis, MO). BP was prepared immediately before use in animal bioassays by dissolving in tricaprylin.

Animals. A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). UL53-3 mice on FVB mouse background, carrying three copies of a transgene containing an Ala-135-Val p53 mutation, were obtained from Dr. Roger Wiseman's group at the National Institute of Environmental Health Sciences, NIH (Research Triangle Park, NC). UL53-3 mice were backcrossed to A/J mice for 10 generations to create the N10 (A/J × UL53-3) mice before their use in the present study. Ink4a/Arf^{KO} mice on a 129-B6 mixed background were obtained from Dr. Ronald A. DePinho's group at Dana Faber Cancer Institute (Boston, MA). The development of the Ink4a/Arf^{KO} mice (knockout mice lacking both *p16INK4a* and *p19ARF* genes) was reported previously (37). Ink4a/Arf^{KO} mice were backcrossed to A/J mice for 6 generations to create the N6 (A/J × Ink4a/Arf^{KO}) mice. All of the bioassays in this study were performed on the F1 mice generated by crossing N10 (A/J × UL53-3) and N6 (A/J × Ink4a/Arf^{KO}) mice. Animals were housed in plastic cages with hardwood bedding and dust covers, in a high efficiency particulate air (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*. For all animal 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 and Ink4a/Arf^{KO} Genotype. All of the animals were genotyped for the p53 mutation (Ala135Val) and Ink4a/Arf^{KO} using the procedures reported previously (16, 31). Briefly, tail clippings from each N10 (A/J × UL53–3) mouse were homogenized and incubated overnight at 37°C in lysis solution [0.4 mg/ml Pronase, 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. The p53 transgene created a restriction fragment length polymorphism with a new *Hph*I restriction enzyme cleavage site (recognition site: GGTGA). This mutation was used to genotype (UL53-3 × A/J)F1 mice using the PCR-restriction fragment length polymorphism method. PCR primers were designed from the regions of mouse p53 exon 5 that contained the Ala-135-Val mutation. The primer sequences were as follows: (*a*) 5′-TAC TCT CCT CCC CTC AAT AAG-3′; and (*b*) 5′-CTC GGG TGG CTC ATA AGG TAC CAC-3′. These PCR primers generated a 190-bp amplified exon 5 fragment from both the *wt p53* allele and transgene allele. After amplification, the fragment was incubated with the restriction endonuclease *Hph*I, which cleaves once within the amplified 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.

For Ink4a/$Arf^{-/-}$ genotype, tail clippings from each mouse were processed for DNA isolation similarly as those described above. PCR primers were derived from the regions of mouse $p16^{INK4a}$ exon 2 and the neo cassette. The primer sequences were as follows: (*a*) $p16^{INK4a}$ exon2F: 5′TTA ACA GCG GAG CTT CGT AC 3′; and (*b*) $p16^{INK4a}$ exon2R: 5′GAA TCT GCA CCG TAG TTG AG 3′. Together, $p16^{INK4a}$ exon2F and $p16^{INK4a}$ exon2R amplify a 159-bp product from the exon 2 of $p16^{INK4a}$. The other pair of primers are: neoF: (*a*) 5′CTT GGG TGG AGA GGC TAT TC 3′ and neoR; and (*b*) 5′AGG TGA GAT GAC AGG AGA TC 3′. Together, neoF and neoR amplify a 280-bp product from the neo insert. The PCR products were then subjected to electrophoresis on a 1% agarose gel, along with a DNA size marker, and visualized by UV light after staining with ethidium bromide. The DNA having both *wt* $p16^{INK4a}$*/ARF* alleles ($Ink4a/Arf^{+/+}$) displayed only a single 159-bp fragment; DNA with a *wt* $p16^{INK4a}$*/ARF* and target mutation allele ($Ink4a/Arf^{+/-}$) showed 159- and 280-bp bands, whereas DNA with both target mutation alleles ($Ink4a/Arf^{-/-}$) showed only a single 280-bp fragment. This procedure was also repeated at least once for confirmation.

Lung Tumorigenesis Studies. Three lung tumor bioassays were conducted simultaneously. For each bioassay, 6-week-old A/J, $p53^{+/+}$Ink4a/$Arf^{+/-}$, $p53^{+/-}$Ink4a/$Arf^{+/+}$, and $p53^{+/-}$Ink4a/$Arf^{+/-}$ mice were randomized into eight groups according to the p53 and Ink4a/Arf genotypes and treatments. As seen in Fig. 1*A* and Table 1, mice in groups 1–4 were given a single i.p. injection of 0.1 ml of tricaprylin as vehicle controls. Mice in groups 4–8 were given a single i.p. injection of BP (100 mg/kg body weight) in 0.1 ml of tricaprylin. Animals were terminated 18 weeks (lung tumor bioassay I), 30 weeks (lung tumor bioassay III), and 40 weeks (lung tumor bioassay II) after exposure to the carcinogen. During the bioassay, 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. At termination, all of the animals were killed by CO_2 asphyxiation. The lungs were fixed in Tellyesniczky's [90% ethanol (70% volume for volume), 5% glacial acetic acid, and 5% formalin (10% volume for volume buffered formalin)] overnight and then kept in 70% ethanol for evaluation before paraffin embedding. Lung tumor development was then evaluated by counting tumor number (N), calculating tumor volume (V) and the total tumor load (total tumor V per mouse). The tumor volumes were determined by measuring the diameter of each tumor, which is generally in a round shape. The radius (r = diameter/2) was determined, and the total tumor volume was calculated by: $V = 4/3\pi r^3$. All available lung tumors were subjected to histopathological examination.

Chemoprevention Studies with Budesonide. Three separate lung tumor chemoprevention studies were performed. For each study, 6-week-old A/J, $p53^{+/+}$Ink4a/$Arf^{+/-}$, $p53^{+/-}$Ink4a/$Arf^{+/+}$, and $p53^{+/-}$Ink4a/$Arf^{+/-}$ mice were randomized into eight groups according to the p53 and Ink4a/Arf genotypes and treatments. As seen in Table 2, mice in groups 1–4 were given a single i.p. injection of BP (100 mg/kg body weight) in 0.1 ml of tricaprylin and fed AIN-76A-purified diet (Dyets, Inc.). Mice in groups 4–8 were given a single i.p. injection of BP (100 mg/kg body weight) in 0.1 ml of tricaprylin and fed AIN-76A-purified diet containing budesonide (1.5 mg/kg diet). As shown in Fig. 1*B*, three study designs were used: (*a*) budesonide was given 2 weeks previous BP treatment and continued for an additional 18 weeks; (*b*) budes-

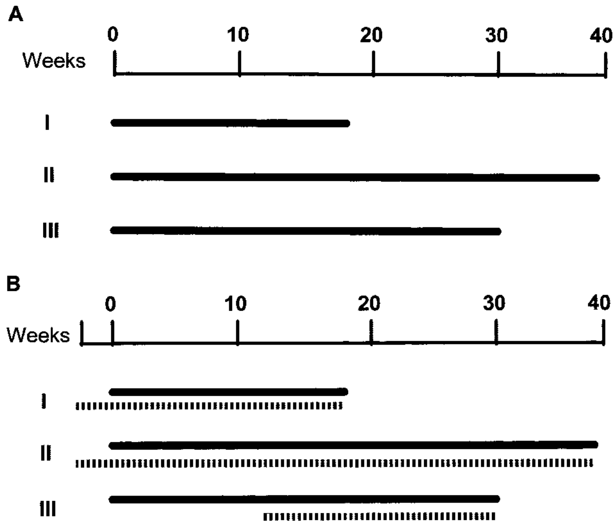

Fig. 1. Experimental design for lung tumorigenesis and chemoprevention by budesonide on BP-induced mouse lung tumorigenesis. *A,* lung tumorigenesis assay with BP. The mice received single i.p. injection of BP at week 0. *Solid horizontal lines,* the time (weeks) for each experiment, 18, 40, and 30 weeks for lung tumorigenesis bioassays 1, 2, and 3, respectively. *B,* chemoprevention study of budesonide on the BP-induced mouse lung tumorigenesis. The mice received single i.p. injection of BP at week 0. *Solid horizontal lines,* the time (by weeks) for each experiment, 18, 40, and 30 weeks for chemoprevention bioassays 1, 2, and 3, respectively. *Shaded lines,* the time (weeks) that mice were on budesonide diet.

onide was given 2 weeks previous BP treatment and continued for an additional 40 weeks; and (*c*) budesonide was given 12 weeks after BP treatment and continued for an additional 18 weeks. Food and water were available *ad libitum*. Lungs were harvested following the same procedure as stated above.

Statistical Analysis. Student's *t* test was used to determine the differences in the number and size of lung tumors per mouse between treatment and control mice. Fisher's exact test was used to determine the difference in the incidence. The significance of percentage changes was calculated using a Z-statistic for proportions

$$\left(Z = \frac{\hat{p}_1 - \hat{p}_2}{\sqrt{\hat{p}(1-\hat{p})(1/n_1 + 1/n_2)}}\right),$$

where $\hat{p}_1$ is the proportional reduction attributable to budesonide in the wt group, $\hat{p}_2$ is the proportional reduction attributable to budesonide in the transgenic groups, $\hat{p}$ is the proportional reduction attributable to budesonide, regardless of genotype, n_1 is the number of wt animals, and n_2 is the number of transgenic animals.

To assess the significance of synergy between the p53 and Ink4a/Arf genotypes at each time point, two-way ANOVA was performed on tumor volume for the 18-, 30-, and 40-week treated mice. Results: 18-week synergy is not significant; 30-week synergy, $P < 0.01$; 40-week synergy, $P < 0.05$.

RESULTS

Synergistic Effect of Germ-line Alterations in p53 and Ink4a/Arf on Carcinogen-induced Mouse Lung Tumorigenesis. Six-week-old A/J, $p53^{+/+}Ink4a/Arf^{+/-}$, $p53^{+/-}Ink4a/Arf^{+/+}$, and $p53^{+/-}Ink4a/Arf^{+/-}$ mice were randomized into eight groups according to the p53 and Ink4a/Arf genotypes and treatments. As seen in Fig. 1*A* and Table 1, mice in groups 1–4 were given a single i.p. injection of 0.1 ml of tricaprylin as vehicle controls. Mice in groups 4–8 were given a single i.p. injection of BP (100 mg/kg body weight) in 0.1 ml of tricaprylin. Animals were terminated 18 weeks after exposure to the carcinogen. At this time, all of lung tumors were diagnosed as lung adenomas. As shown in Table 1, $p53^{+/-}Ink4a/Arf^{+/+}$ and $p53^{+/-}Ink4a/Arf^{+/-}$ mice carrying a mutant p53 transgene (Val135) with or without Ink4a/Arf heterozygous deletion developed a higher number of lung tumors (an average of 12.5 tumors/mouse) with larger size (~7.5 mm^3) after treatment with BP than wt and $p53^{+/+}Ink4a/Arf^{+/-}$ mice (an average of 7.5 tumors/mouse; ~3 mm^3; $P < 0.001$; Table 1). In the vehicle control groups, a low incidence of lung tumors was observed, and there was no significant difference in tumor multiplicity among the four groups. In general, p53 mutation caused an increase in both tumor multiplicity and size, whereas Ink4a/Arf heterozygous deficiency did not

Table 1 *Mouse lung tumorigenesis induced by a BP-induced lung tumorigenesis in A/J mice bearing a dominant negative mutant p53 and/or heterozygous deficiency for ink4a/Arf*

Weeks post-BP	Group	No. of mice	Genotype	Treatment[a]	Lung tumor incidence	Tumor no./mouse	Tumor vol. (mm^3)	Increase in no. (%)	Increase in vol. (%)
18	1	15	$p53^{+/+}Ink4a/Arf^{+/+}$	Vehicle	0/17 (0%)	0.00 ± 0.00	0.00 ± 0.00		
	2	16	$p53^{+/+}Ink4a/Arf^{+/-}$	Vehicle	1.18 (5.5%)	0.06 ± 0.24	0.03 ± 0.12		
	3	16	$p53^{+/-}Ink4a/Arf^{+/+}$	Vehicle	2/18 (11.1%)	0.11 ± 0.32	0.04 ± 0.14		
	4	16	$p53^{+/-}Ink4a/Arf^{+/-}$	Vehicle	2/18 (11.1%)	0.11 ± 0.32	0.08 ± 0.24		
	5	13	$p53^{+/+}Ink4a/Arf^{+/+}$	BP	13/13 (100%)	7.38 ± 5.44	3.21 ± 2.77	100	100
	6	13	$p53^{+/+}Ink4a/Arf^{+/-}$	BP	13/13 (100%)	7.83 ± 4.73	2.96 ± 2.08	106	92
	7	12	$p53^{+/-}Ink4a/Arf^{+/+}$	BP	12/12 (100%)	12.33 ± 6.27[b]	7.29 ± 3.98[b]	167	226
	8	12	$p53^{+/-}Ink4a/Arf^{+/-}$	BP	12/12 (100%)	12.58 ± 5.09[b]	7.62 ± 5.79[b]	170	237
30	1	20	$p53^{+/+}Ink4a/Arf^{+/+}$	Vehicle	6/20 (30.0%)	0.29 ± 0.46	0.81 ± 2.51		
	2	18	$p53^{+/+}Ink4a/Arf^{+/-}$	Vehicle	5/18 (27.8%)	0.41 ± 0.63	11.19 ± 47.35		
	3	21	$p53^{+/-}Ink4a/Arf^{+/+}$	Vehicle	11/21 (52.4%)	0.50 ± 0.51	6.13 ± 18.12		
	4	18	$p53^{+/-}Ink4a/Arf^{+/-}$	Vehicle	8/18 (44.4%)	1.00 ± 1.88	6.08 ± 16.92		
	5	48	$p53^{+/+}Ink4a/Arf^{+/+}$	BP	48/48 (100%)	12.24 ± 4.26	17.19 ± 9.99	100	100
	6	43	$p53^{+/+}Ink4a/Arf^{+/-}$	BP	43/43 (100%)	12.57 ± 4.75	25.03 ± 14.21[b]	103	146
	7	35	$p53^{+/-}Ink4a/Arf^{+/+}$	BP	35/35 (100%)	20.72 ± 5.21[b]	159.17 ± 170.12[b]	169	926
	8	19	$p53^{+/-}Ink4a/Arf^{+/-}$	BP	19/19 (100%)	22.35 ± 5.27[b]	414.59 ± 359.07[b,c]	183	2412
40	1	16	$p53^{+/+}Ink4a/Arf^{+/+}$	Vehicle	5/16 (31.3%)	0.31 ± 0.47	1.24 ± 3.27		
	2	17	$p53^{+/+}Ink4a/Arf^{+/-}$	Vehicle	7/17 (41.2%)	0.47 ± 0.62	5.76 ± 15.76		
	3	15	$p53^{+/-}Ink4a/Arf^{+/+}$	Vehicle	7/15 (46.7%)	0.53 ± 0.64	2.10 ± 4.61		
	4	14	$p53^{+/-}Ink4a/Arf^{+/-}$	Vehicle	6/14 (42.9%)	1.36 ± 2.37	10.67 ± 22.27		
	5	12	$p53^{+/+}Ink4a/Arf^{+/+}$	BP	12/12 (100%)	11.63 ± 3.20	24.09 ± 15.7	100	100
	6	15	$p53^{+/+}Ink4a/Arf^{+/-}$	BP	15/15 (100%)	12.00 ± 2.98	103.40 ± 123.13[d]	103	429
	7	16	$p53^{+/-}Ink4a/Arf^{+/+}$	BP	16/16 (100%)	23.31 ± 5.80[b]	218.29 ± 200.31[b]	200	906
	8	6	$p53^{+/-}Ink4a/Arf^{+/-}$	BP	6/6 (100%)	27.00 ± 1.55[b]	552.39 ± 323.65[b,c]	232	2293

[a] At 6 weeks of age, mice were given single i.p. injections of BP at a dose of 100 mg/kg in tricaprylin, which was counted as week 0. Mice in all experiments (1, 2, and 3; Fig. 1*A*) were fed with AIN76A diet. The mice were terminated at weeks 18, 30, and 40, respectively. Approximately equal numbers of males and females were used, with no significant difference in tumor multiplicity between the sexes.

[b] $P < 0.01$, tumor multiplicity or tumor volume in mutant p53 and/or heterozygous deficient of Ink4a/Arf mice groups was significantly different from their wt littermates.

[c] $P < 0.01$ (30 weeks) and $P < 0.05$ (40 weeks) for synergistic effect between alterations in p53 and Ink4a/Arf.

[d] $P < 0.05$, tumor volume in heterozygous deficient Ink4a/Arf mice group was significantly different from their wt littermates.

Table 2 *Chemopreventive efficacy of budesonide on BP-induced lung tumorigenesis in A/J mice bearing a dominant negative mutant p53 and/or heterozygous deficiency for Ink4a/Arf*

Experiment	Group	No. of mice	Genotype	Treatment[a]	Lung tumor incidence	Tumor no./mouse	Tumor vol. (mm^3)	Decrease in no.	Decrease in vol.
I	1	13	p53$^{+/+}$Ink4a/Arf$^{+/+}$	BP	13/13 (100%)	7.38 ± 5.44	3.21 ± 2.77		
	2	13	p53$^{+/+}$Ink4a/Arf$^{+/-}$	BP	13/13 (100%)	7.83 ± 4.73	2.96 ± 2.08		
	3	12	p53$^{+/-}$Ink4a/Arf$^{+/+}$	BP	12/12 (100%)	12.33 ± 6.27	7.29 ± 3.98		
	4	12	p53$^{+/-}$Ink4a/Arf$^{+/-}$	BP	12/12 (100%)	12.58 ± 5.09	7.62 ± 5.79		
	5	8	p53$^{+/+}$Ink4a/Arf$^{+/+}$	BP/Bud	5/8 (62.5%)	1.38 ± 1.69[b]	0.39 ± 0.84[b]	81.3%	87.6%
	6	8	p53$^{+/+}$Ink4a/Arf$^{+/-}$	BP/Bud	4/8 (50.0%)	0.63 ± 0.74[b]	0.17 ± 0.33[b]	92.0%	94.3%
	7	8	p53$^{+/-}$Ink4a/Arf$^{+/+}$	BP/Bud	7/8 (87.5%)	1.71 ± 2.36[b]	0.62 ± 1.19[b]	86.1%	91.5%
	8	8	p53$^{+/-}$Ink4a/Arf$^{+/-}$	BP/Bud	7/8 (87.5%)	2.38 ± 3.16[b]	0.38 ± 0.32[b]	81.1%	95.0%
II	1	12	p53$^{+/+}$Ink4a/Arf$^{+/+}$	BP	12/12 (100%)	11.63 ± 3.20	24.09 ± 15.7		
	2	15	p53$^{+/+}$Ink4a/Arf$^{+/-}$	BP	15/15 (100%)	12.00 ± 2.98	103.40 ± 123.13		
	3	16	p53$^{+/-}$Ink4a/Arf$^{+/+}$	BP	16/16 (100%)	23.31 ± 5.80	218.29 ± 200.31		
	4	6	p53$^{+/-}$Ink4a/Arf$^{+/-}$	BP	6/6 (100%)	27.00 ± 1.55	552.39 ± 323.65		
	5	19	p53$^{+/+}$Ink4a/Arf$^{+/+}$	BP/Bud	17/19 (90%)	3.47 ± 2.26[b]	1.51 ± 1.58[b]	69.8%	94%[c]
	6	11	p53$^{+/+}$Ink4a/Arf$^{+/-}$	BP/Bud	8/11 (73%)	2.72 ± 3.03[b]	1.71 ± 2.56[b]	77.5%	98%
	7	18	p53$^{+/-}$Ink4a/Arf$^{+/+}$	BP/Bud	18/18 (100%)	5.44 ± 2.33[b]	12.54 ± 15.61[b]	76.5%	94%
	8	5	p53$^{+/-}$Ink4a/Arf$^{+/-}$	BP/Bud	5/5 (100%)	9.20 ± 3.70[b]	125.83 ± 79.76[b]	65.9%	77%[c]
III	1	48	p53$^{+/+}$Ink4a/Arf$^{+/+}$	BP	48/48 (100%)	12.24 ± 4.26	17.19 ± 9.99		
	2	43	p53$^{+/+}$Ink4a/Arf$^{+/-}$	BP	43/43 (100%)	12.57 ± 4.75	25.03 ± 14.21		
	3	35	p53$^{+/-}$Ink4a/Arf$^{+/+}$	BP	35/35 (100%)	20.72 ± 5.21	159.17 ± 170.12		
	4	19	p53$^{+/-}$Ink4a/Arf$^{+/-}$	BP	19/19 (100%)	22.35 ± 5.27	414.59 ± 359.07		
	5	12	p53$^{+/+}$Ink4a/Arf$^{+/+}$	BP/Bud	12/12 (100%)	5.70 ± 1.83[b]	3.13 ± 1.65[b]	53.4%	81.8%[d]
	6	8	p53$^{+/+}$Ink4a/Arf$^{+/-}$	BP/Bud	8/8 (100%)	8.00 ± 2.83[b]	8.97 ± 3.69[b]	36.4%	64.2%
	7	10	p53$^{+/-}$Ink4a/Arf$^{+/+}$	BP/Bud	10/10 (100%)	11.44 ± 3.47[b]	87.11 ± 107.72[b]	44.8%	44.6%
	8	6	p53$^{+/-}$Ink4a/Arf$^{+/-}$	BP/Bud	6/6 (100%)	11.20 ± 4.92[b]	278.57 ± 173.83[b]	49.9%	32.8%[d]

[a] At 6 weeks of age, mice were given single i.p. injections of BP at a dose of 100 mg/kg in tricaprylin, which was counted as week 0. Mice in experiments 1 and 2 were fed with either AIN76A diet or budesonide (1.5 mg/kg) diet 2 weeks before the BP treatment and continued to weeks 18 and 40, respectively. Mice in experiment 3 were fed with either AIN76A diet or budesonide (1.5 mg/kg) diet 12 weeks post-BP treatment and continued to week 30. Approximately equal numbers of males and females were used, with no significant difference in tumor multiplicity between the sexes.

[b] $P < 0.01$, tumor multiplicity or volume in budesonide groups was significantly different from BP treatment groups.

[c] $P = 0.07$, tumor volume was decreased in p53$^{+/-}$Ink4a/Arf$^{+/-}$ mice as compared with their wt littermates.

[d] $P < 0.01$, tumor volume was decreased in p53$^{+/-}$Ink4a/Arf$^{+/-}$ mice as compared with their wt littermates.

have any effect on either tumor multiplicity or size at this stage of lung tumorigenesis, which is consistent with our previous observations (16).

The designs of the second and third bioassays were similar to the first bioassay with the exception of the termination time point. Animals were terminated 40 weeks after exposure to BP for the second bioassay and 30 weeks for the third bioassay. Both of these bioassays were designed to determine the effect of the p53 and Ink4a/Arf on lung tumor progression (Fig. 1*A* and Table 1). The incidence of lung tumors in all four groups of treated mice was 100%. Similar to the first lung tumor bioassay, p53$^{+/-}$Ink4a/Arf$^{+/+}$ and p53$^{+/-}$Ink4a/Arf$^{+/-}$ mice developed a higher number of lung tumors (an average of 21 tumors/mouse for bioassay 3 and an average of 25 tumors/mouse for bioassay 2) after treatment with BP than wt and p53$^{+/+}$Ink4a/Arf$^{+/-}$ mice (an average of 12.5 tumors/mouse for bioassay 3 and an average of 12 tumors/mouse for bioassay 2). More interestingly, p53$^{+/-}$Ink4a/Arf$^{+/-}$ mice exhibited a striking increase in tumor volume (~24-fold in bioassay 3 and ~23-fold in bioassay 2) compared with a 9-fold increase in tumor volume in mice with only the p53 dominant negative mutation (p53$^{+/-}$Ink4a/Arf$^{+/+}$). There was also a ~50% and a ~4-fold increase in tumor volume in Ink4a/Arf heterozygous-deficient mice (p53$^{+/+}$Ink4a/Arf$^{+/-}$) in bioassays 3 and 2, respectively, indicating that the effect of Ink4A/Arf heterozygous deficiency is mostly on late stage lung tumor progression.

In addition, most of the lung tumors (~80%) from mice with a p53 mutation and deletion of Ink4A/Arf (p53$^{+/-}$Ink4a/Arf$^{+/-}$) were lung adenocarcinomas. In contrast, lung adenocarcinomas were seen in <10% of the lung tumors from the wt mice and ~50% of the lung tumors from either p53 transgenic mice or Ink4a/Arf heterozygous deficient mice. Fig. 2 shows the gross photomicrographs of lung tumors from mice with various genotypes. Lung tumors were significantly larger in mice treated with BP alone (Fig. 2, *A–D*) than those treated with BP and budesonide (Fig. 2, *E–H*). Fig. 3 shows the light

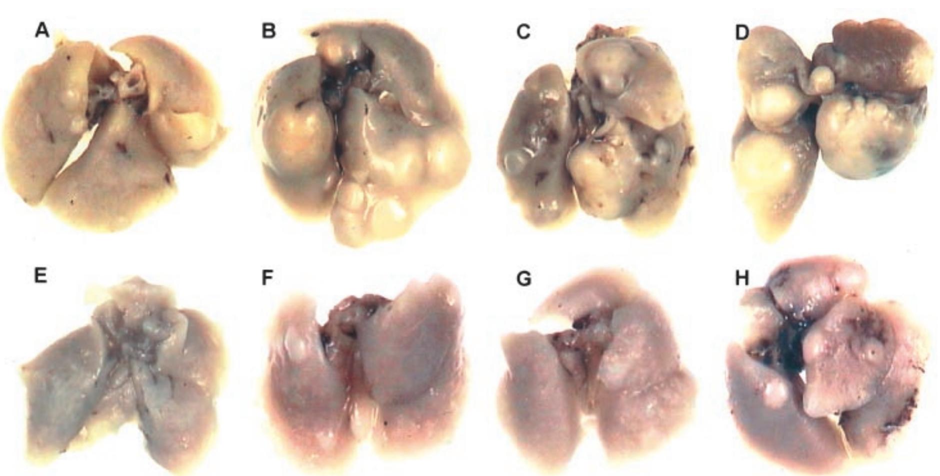

Fig. 2. Lung tumors from p53$^{+/-}$Ink4a/Arf$^{+/-}$ mice. *A–D*, gross photomicrographs of lungs from p53$^{+/+}$Ink4a/Arf$^{+/+}$, p53$^{+/-}$Ink4a/Arf$^{+/+}$, p53$^{+/+}$Ink4a/Arf$^{+/-}$, and p53$^{+/-}$Ink4a/Arf$^{+/-}$, respectively, 40 weeks after BP treatment. *E–H*, lungs from p53$^{+/+}$Ink4a/Arf$^{+/+}$, p53$^{+/-}$Ink4a/Arf$^{+/+}$, p53$^{+/+}$Ink4a/Arf$^{+/-}$, and p53$^{+/-}$Ink4a/Arf$^{+/-}$, respectively, 40 weeks after BP and budesonide treatment.

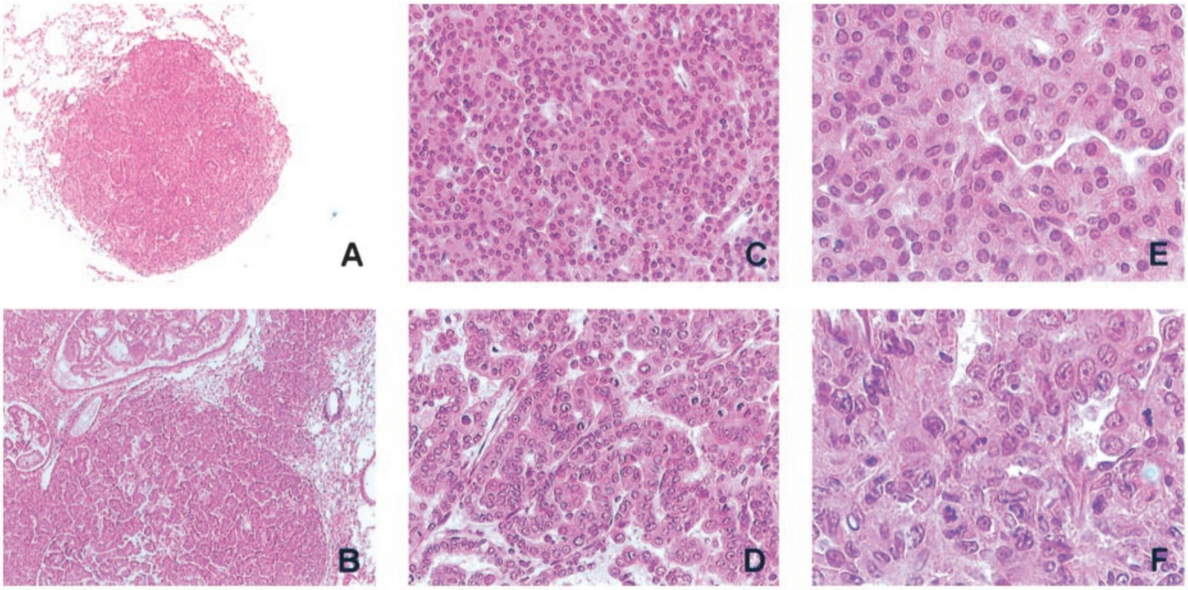

Fig. 3. BP-induced lung tumors in A/J mice carrying a dominant negative p53 and heterozygous deletion of Ink4a/Arf. *A* and *B*, light photomicrographs of lung tumors (adenoma *versus* carcinoma) at ×4 magnifications; *C* and *D*, lung tumors at ×20 magnifications; *E* and *F*, lung tumors at ×40 magnifications from $p53^{+/+}$Ink4a/$Arf^{+/+}$ and $p53^{+/-}$Ink4a/$Arf^{+/-}$ mice, respectively.

photomicrographs of lung tumors (adenoma *versus* carcinoma) from mice with a dominant negative p53 and/or heterozygous deletion of Ink4a/Arf. As shown in Fig. 3, *B*, *D*, and *F*, mouse lung adenocarcinomas are composed of cells with varying degrees of differentiation with most cells appearing relatively undifferentiated relative to lung adenomas (Fig. 3, *A*, *C*, and *E*). There is a complete loss of normal alveolar architecture, and the nuclear:cytoplasmic ratio is increased in the carcinomas (Fig. 3, *B*, *D*, and *F*). Nuclear crowding and cytologic atypia are present, and there is heterogeneity of growth patterns. Invasion into adjacent bronchioles or vessels is also observed (Fig. 3*B*). These results indicate a significant synergistic interaction between the presence of a mutant p53 transgene and the Ink4A/Arf deletion during lung tumor progression.

Differential Chemopreventive Effects of Budesonide on Carcinogen-induced Mouse Lung Tumorigenesis in wt and Mutant A/J Mice. Three separate lung tumor chemoprevention studies using budesonide were performed. The first two chemoprevention studies, budesonide (1.5 mg/kg diet), was administered beginning 2 weeks before BP administration and given continually for 18 weeks (chemoprevention study 1 for short-term effect) or for 40 weeks (chemoprevention study 2 for long-term effect).

In the first chemoprevention study, treatment with budesonide reduced tumor multiplicity by 81–92% ($P < 0.01$) and size by 87–95% ($P < 0.01$) in all four genotypic groups (Fig. 1*B* and Table 2). This represents a significant inhibition of lung tumor multiplicity and size in the early stages of mouse lung tumorigenesis, regardless of germ-line alterations in either p53 and/or Ink4A/Arf. In the second chemoprevention study in which animals were terminated 40 weeks after BP treatment, treating mice with budesonide reduced tumor multiplicity by an average of 70% ($P < 0.01$) in all four genotypic groups when compared with those from the same genotypic groups treated with BP alone. Similarly, treatment with budesonide reduced tumor size by 94, 98, and 94% in wt, $p53^{+/+}$Ink4a/$Arf^{+/-}$ mice, and $p53^{+/-}$Ink4a/$Arf^{+/+}$ mice, respectively, when compared with those from the same genotypic groups treated with BP alone (Table 2). In contrast, inhibition of lung tumor size was only 77%; inhibition of lung tumor size was observed in $p53^{+/-}$Ink4a/$Arf^{+/-}$ mice treated with budesonide ($P = 007$; Table 2), suggesting a decrease in efficacy of budesonide against late stage lung tumorigenesis in mice with multiple genetic defects.

The third chemoprevention study was conducted, with budesonide given 12 weeks after BP treatment and continued for an additional 18 weeks to determine the effect of budesonide on established lung lesions, because most mice exhibited multiple small lung adenomas by 18 weeks. As shown in Table 2, inhibition of tumor multiplicity was roughly 65–77% in all four genotypes without significant genotype-specific variations. However, the degree of budesonide chemopreventive effect on tumor size was profoundly dependent on mouse genotypes. Thus, mice with wt, $p53^{+/+}$Ink4a/$Arf^{+/-}$, $p53^{+/-}$Ink4a/$Arf^{+/+}$, and $p53^{+/-}$Ink4a/$Arf^{+/-}$ showed 82, 64, 45, and 33% decrease in tumor volume, respectively. The difference between 82% inhibition and 33% inhibition is statistically significant ($P < 0.01$; Table 2). The mutant mice with alterations in p53 and/or Ink4A/Arf seemed to exhibit a significant resistance to chemoprevention by budesonide. This observation has immediate clinical implications in at least two aspects: (*a*) the third chemoprevention study may more closely parallel potential clinical trials by exposing individuals with established precancerous lesions; and (*b*) budesonide may be much less efficacious in lesions that contain alterations in p53 and Ink4a/Arf.

DISCUSSION

In this study, we described two major findings: (*a*) we demonstrated a significant synergistic effect of germ-line alterations in p53 and Ink4a/Arf on lung tumor progression; and (*b*) we observed differential chemopreventive effects of budesonide on BP-induced lung tumorigenesis in wt and mutant mice with alterations in p53 and/or Ink4a/Arf, which was particularly striking when budesonide treatment was initiated later. We have clearly demonstrated the functional role of p53 and Ink4a/Arf on lung tumorigenesis. The resulting tumor model for human adenocarcinoma, namely p53 transgenic and Ink4a/Arf heterozygous deficient mice on an A/J background, includes alterations in commonly altered genes, *e.g.*, Kras2, p53, and Ink4a/Arf when treated with lung-specific chemical carcinogens. Clearly, this mouse model is superior over the commonly used A/J mice in examining lung adenocarcinomas.

Mutation or deletions of the Kras2, p53, and Ink4a/Arf tumor suppressor genes are the most common genetic defect detected in human lung cancer. p53 mutations have been found in 50–80% of sporadic lung cancers, indicating that p53 plays a crucial role in the development of human lung cancer (38). Thus, mouse lung tumor models with germ-line p53 mutations would be important for determination of the role of p53 in lung tumorigenesis, *e.g.*, our model is directly related to patients with LFS who develop lung cancer at a remarkably high rate.[5] 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.[5] Although 10% of smokers develop lung cancer, ~50% of males with LFS develop lung cancer. In a previous study, we found that (UL53-3 × A/J) F1 p53 transgenic mice carrying a p53 transgene (Val135) exhibit an increased susceptibility to early stage lung tumor development with animals terminated 16 weeks after carcinogen treatment, suggesting that inactivation of p53 is involved in the early stage of lung tumorigenesis (16). The present study demonstrated that mice with a germline p53 mutation also developed a significant increase in lung tumor progression, indicating that the p53 mutation not only plays a role in early stage but also in late stage of the carcinogenic process.

p16 or INK4A is another tumor suppressor that is frequently inactivated in human lung cancer. Ng *et al.* (39) investigated methylated p16 in patients with NSCLC and found that the patients with plasma and preresection pleural lavage methylated p16 have shorter survival. Patients with $p16^{1NK4A}$-negative tumors had a significantly shorter survival (4 months) than those with $p16^{INK4A}$ protein expression (15 months). Jin *et al.* (40) have studied the association of the immunohistochemical expressions of cyclin D1, p16, and pRB with the prognoses of 106 patients with NSCLC at stages I and II after complete resection was investigated. Their results indicate that cyclin D1 and p16, especially a combination of cyclin D1 and p16, are very useful in predicting the prognosis of a patient with NSCLC after curative resection, independent of pathological stages I and II (40). Similarly, mice with a heterozygous deficiency for Ink4a/Arf were significantly more susceptible to mouse lung tumor progression (31). The results from the present study extend our previous observations that mice with a heterozygous deficiency of Ink4a/Arf exhibited a striking enhancement in lung tumor progression at 30 and 40 weeks after carcinogen treatment when compared with their respective wt mice, whereas mice with Ink4a/Arf heterozygous deficiency showed little effect on early stages of lung tumor development.

One of the most interesting results is the observation that mutant *p53* acts synergistically with deletion of Ink4a/Arf to accelerate the development of undifferentiated malignant lung tumors. In fact, mice with p53 dominant negative mutation and Ink4a/Arf heterozygous deficiency exhibited a >20-fold increase in tumor volume compared with a 4-fold increase in Ink4a/Arf heterozygous deficient mice and a 9-fold increase in mice with only the p53 dominant negative mutation. This synergistic effect occurs at the stage of tumor progression because most of lung tumors (~80%) from mice with p53 mutation and deletion of Ink4a/Arf ($p53^{+/-}$Ink4a/$Arf^{+/-}$) were lung adenocarcinomas compared with 10% in wt mice and 50% in Ink4a/Arf heterozygous deficient mice. The synergistic relationship between alterations of the $p16^{INK4A}$ and *p53* genes in tumor development has recently been reported (41, 42), *e.g.*, Kinoshita *et al.* (41) while examining NSCLC in humans have shown that proliferative activity was considerably higher in p53 mutant than normal tumors, and loss of p16 expression was associated with a further increase in proliferative activity in the p53 mutant tumors but not with proliferative activity in the p53-negative tumors. Bardeesy *et al.* (42) created compound mutants of inactivated p16 and mutant p53 in transforming growth factor-α transgenic mice and demonstrated a synergistic interaction between inactivated p16 and mutant p53 as the transforming growth factor-α animals heterozygous for both the Ink4a/Arf and p53 mutation showed a dramatically increased incidence of serous cystadenoma than those with heterozygosity at either locus alone.

In the present study, we have examined the use of these models in the chemoprevention studies using budesonide. The efficacy of budesonide in these models was examined because the altered *p53* and *Ink4a/Arf* appear particularly relevant to human lung cancer but are not routinely altered in carcinogen-induced lung tumors in A/J mice until late in tumor development, if at all. These models are particularly relevant because many early preinvasive lesions of the human aerodigestive tract have alterations in both p53 and p16. On the other hand, our mouse models closely resemble the A/J mouse lung tumor model, because our mice have a pure A/J background with either p53 dominant negative mutation $p53^{val135/wt}$, heterozygous deletion of Ink4a/Arf, or both. Early and continual budesonide administration in the diet exhibited a potent protective effect against BP-induced lung tumorigenesis in mice of different genotypes particularly at the 18-week time point. Budesonide produced a striking 70–80% reduction in tumor multiplicity and an 88–95% in total tumor volume in the short-term (18 week) study irrespective of the genotype. This is consistent with previous studies using either A/J mice or mice with a p53 mutation (2, 3). Similarly, in mice exposed to budesonide continually and sacrificed at 40 weeks, we found a consistent decrease in tumor multiplicity (66–76%) and a decrease in tumor volume of 94–98% in wt, p53 mutant, or Ink4a/Arf-deficient mice. In contrast, mice with both a p53 mutation and Ink4a/Arf deficiency showed only a 77% decrease in tumor volume. Most strikingly, however, in the late treatment experiment in which mice were treated with budesonide for 12 weeks after they had developed small adenomas, one observed a ~40–50% decrease in tumor multiplicity in all groups of animals. However, when examining effects on tumor volume, we observed a striking genotype-dependent effect with wt mice, $p53^{+/+}$Ink4a/$Arf^{+/-}$ mice, $p53^{+/-}$Ink4a/$Arf^{+/+}$ mice, and $p53^{+/-}$Ink4a/$Arf^{+/-}$ showing 82, 64, 45, and 33% decreases in tumor volume, respectively. Thus, mice with genetic alterations of p53 and Ink4a/Arf are highly resistant to budesonide compared with wt mice during late intervention.

The mechanism for the observed differential chemopreventive effect of budesonide during late intervention is not clear at present. Two general mechanisms for the inhibitory effect of budesonide on cancer have been proposed, namely, induction of p27/p21 or induction of apoptosis (43–47). A recent study has shown that cells treated with corticosterone have an increased binding of p27 with CDK4, with a strong decrease in the kinase activity of the CDK4–cyclin D1 complex, suggesting that induction of p27/p21 may play a role in growth inhibition by corticosterone (43, 47). Glucocorticoids have also been shown to induce G_1 arrest and apoptosis of several leukemia cell lines (44–46). It is likely that some of the budesonide-mediated chemopreventive effects would require the presence of either wt p53, wt Ink4a/Arf, or both. Because mutant A/J mice are the most likely to closely resemble the human counterpart than wt A/J mice, our study indicates that budesonide will be less effective in inhibiting or regressing human lung precancer lesions than might be predicted through the use of A/J mice (2, 3). Thus, the models described here would appear particularly relevant to examining potential chemopreventive and therapeutic agents using an *in situ* model with a number of the known mutations relevant to lung adenocarcinomas in humans.

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REFERENCES

1. Minna, J. D. The molecular biology of lung cancer pathogenesis. Chest, *103:* 449–456, 1993.
2. You, M., and Bergman, G. Preclinical and clinical models of lung cancer chemoprevention. Hematol. Oncol. Clin. N. Am., *12:* 1037–1053, 1998.
3. Herzog, C. R., Lubet, R. A., and You, M. Genetic epigenetic alterations in mouse lung tumors: implications for cancer chemoprevention. J. Cell. Biochem., *28/29S:* 49–63, 1997.
4. You, M., Candrian, U., Maronpot, R., Stoner, G., and Anderson, M. Activation of the K-ras protooncogenes in spontaneously occurring and chemically induced lung tumors of the strain A mouse. Proc. Natl. Acad. Sci. USA, *86:* 3070–3074, 1989.
5. Chen, B., Johanson, L., Wiest, J., Anderson, M., and You, M. The second intron of the K-ras gene contains regulatory elements associated with mouse lung tumor susceptibility. Proc. Natl. Acad. Sci. USA, *91:* 1589–1593, 1994.
6. Stowers, S., Gloves, P., Reynolds, S., Maronpot, R., Boone, L., Aaronson, S., and Anderson, M. Activation of the K-ras proto-oncogene in lung tumors form rats and mice chronically exposed to tetranitromethane. Cancer Res., *47:* 3212–3219, 1987.
7. Devereux, T., Anderson, M., and Belinsky, S. Role of ras protooncogene activation in the formation of spontaneous and nitrosamine-induced lung tumors in the resistant C3H mouse. Carcinogenesis (Lond.), *12:* 299–303, 1991.
8. Hegi, M. E., Devereux, T. R., Dietrich, W. F., Cochran, C. J., Lander, E. S., Foley, J. F., Maronpot, R. R., Anderson, M. W., and Wiseman, R. W. Allelotype analysis of mouse lung carcinomas reveals frequent allelic losses on chromosome 4 and an association between allelic imbalances on chromosome 6 and K-ras activation. Cancer Res., *54:* 6257–6264, 1994.
9. Zhang, Z., Wang, Y., Vikis, H. G., Johnson, L., Liu, G., Li, J., Anderson, M. W., Sills, R. C., Hong, H. L., Devereux, T. R., Jacks, T., Guan, K. L., and You, M. Wild-type K-ras can inhibit lung carcinogenesis in mice. Nat. Genet., *29:* 25–33, 2001.
10. Chompret, A. The Li-Fraumeni syndrome. Biochimie, *84:* 75–82, 2002.
11. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature (Lond.), *356:* 215–221, 1992.
12. Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. Tumor spectrum analysis in p53-mutant mice. Curr. Biol., *4:* 1–7, 1994.
13. Donehower, L. A. The p53-deficient mouse: a model for basic and applied cancer studies. Semin. Cancer Biol., *7:* 269–278, 1996.
14. Harvey, M., McArthur, M. J., Montgomery, C. A., Butel, J. S., Bradley, A., and Donehower, L. A. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat. Genet., *5:* 225–229, 1993.
15. Lavigueur, A., Maltby, V., Mock, D., Rossant, J., Pawson, T., and Bernstein, A. High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Mol. Cell. Biol., *9:* 3982–3991, 1989.
16. Zhang, Z., Liu, Q., Lantry, L. E., Wang, Y., Kelloff, G. J., Anderson, M. W., Wiseman, R. W., Lubet, R. A., and You, M. 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. Cancer Res., *60:* 901–907, 2000.
17. Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E., and Skolnick, M. H. A cell cycle regulator involved in genesis of many tumor types. Science (Wash. DC), *264:* 436–440, 1994.
18. Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell, *83:* 993–1000, 1995.
19. Serrano, M., Hannon, G., and Beach, D. A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. Nature (Lond.), *366:* 704–707, 1993.
20. Otterson, G. A., Kratzke, R. A., Coxon, A., Kim, Y. W., and Kaye, F. J. Absence of p16INK4 protein is restricted to the subset of lung cancer lines that retains wildtype RB. Oncogene, *9:* 3375–3378, 1994.
21. Kelley, M. J., Nakagawa, K., Steinberg, S. M., Mulshine, J. L., Kamb, A., and Johnson, B. E. Differential inactivation of CDKN2 and Rb protein in non-small-cell and small-cell lung cancer cell lines. J. Natl. Cancer Inst. (Bethesda), *17:* 756–761, 1995.
22. Hamada, K., Kohno, T., Kawanishi, M., Ohwada, S., and Yokota, J. Association of CDKN2A(p16)/CDKN2B(p15) alterations and homozygous chromosome arm 9p deletions in human lung carcinoma. Genes Chromosomes Cancer, *22:* 232–240, 1998.
23. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat. Med., *1:* 686–692, 1995.
24. Gazzeri, S., Gouyer, V., Vour'ch, C., Brambilla, C., and Brambilla, E. Mechanisms of p16INK4A inactivation in non small-cell lung cancers. Oncogene, *16:* 497–504, 1998.
25. Honda, R., and Yasuda, H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. EMBO J., *18:* 22–27, 1999.
26. Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. Nucleolar Arf sequesters Mdm2 and activates p53. Nat. Cell. Biol., *1:* 20–26, 1999.
27. Zhang, Y., Xiong, Y., and Yarbrough, W. G. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell, *92:* 725–734, 1998.
28. Zindy, F., Eischen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J., and Roussel, M. F. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev., *12:* 2424–2433, 1998.
29. Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., Cordon-Cardo, C., and DePinho, R. A. Cell, *92:* 713–723, 1998.
30. Sanchez-Cespedes, M., Reed, A. L., Buta, M., Wu, L., Westra, W. H., Herman, J. G., Yang, S. C., Jen, J., and Sidransky, D. Inactivation of the INK4A/ARF locus frequently coexists with TP53 mutations in non-small cell lung cancer. Oncogene, *18:* 5843–5849, 1999.
31. Zhang, Z., Wang, Y., Herzog, C. R., Liu, G., Lee, H., DePinho, R. A., and You, M. A strong candidate gene for the Papg1 locus on mouse chromosome 4 affecting lung tumor progression. Oncogene, *21:* 5960–5966, 2002.
32. Shimkin, M. B., and Stoner, G. D. Lung tumors in mice: application to carcinogenesis bioassay. Adv. Cancer Res., *21:* 1–58, 1975.
33. Wattenberg, L. W., and Estensen, R. D. Chemopreventive effects of myo-inositol and dexamethasone on benzo[a]pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary carcinogenesis in female A/J mice. Cancer Res., *56:* 5132–5135, 1996.
34. Wattenberg, L. W., and Estensen, R. D. Studies of chemopreventive effects of budesonide on benzo[a]pyrene-induced neoplasia of the lung and forestomach of female A/J mice. Carcinogenesis (Lond.), *18:* 2015–2017, 1997.
35. Wattenberg, L. W., Wiedmann, T. S., Estensen, R. D., Zimmerman, C. L., Steele, V. E., and Kelloff, G. K. Chemoprevention of pulmonary carcinogenesis by aerosolized budesonide in female A/J mice. Cancer Res., *57:* 5489–5492, 1997.
36. Wattenberg, L. W., Wiedmann, T. S., Estensen, R. D., Zimmerman, C. L., Galbraith, A. R., Steele, V. E., and Kelloff, G. J. Chemoprevention of pulmonary carcinogenesis by brief exposures to aerosolized budesonide or beclomethasone dipropionate and by the combination of aerosolized budesonide and dietary myo-inositol. Carcinogenesis (Lond.), *21:* 179–182, 2000.
37. Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R. A. Role of the INK4a locus in tumor suppression and cell mortality. Cell, *85:* 27–37, 1996.
38. Salgia, R., and Skarin, A. T. Molecular abnormalities in lung cancer. J. Clin. Oncol., *16:* 1207–1217, 1998.
39. Ng, C. S., Zhang, J., Wan, S., Lee, T. W., Arifi, A. A., Mok, T., Lo, D. Y., and Yim, A. P. Tumor p16M is a possible marker of advanced stage in non-small cell lung cancer. J. Surg. Oncol., *79:* 101–106, 2002.
40. Jin, M., Inoue, S., Umemura, T., Moriya, J., Arakawa, M., Nagashima, K., and Kato, H. Cyclin D1, p16 and retinoblastoma gene product expression as a predictor for prognosis in non-small cell lung cancer at stages I and II. Lung Cancer, *34:* 207–218, 2001.
41. Kinoshita, I., Dosaka-Akita, H., Mishina, T., Akie, K., Nishi, M., Hiroumi, H., Hommura, F., and Kawakami, Y. Altered p16INK4 and retinoblastoma protein status in non-small cell lung cancer: potential synergistic effect with altered p53 protein on proliferative activity. Cancer Res., *56:* 5557–5562, 1996.
42. Bardeesy, N., Morgan, J., Sinha, M., Signoretti, S., Srivastava, S., Loda, M., Merlino, G., and DePinho, R. A. Obligate roles for p16(Ink4a) and p19(Arf)-p53 in the suppression of murine pancreatic neoplasia. Mol. Cell. Biol., *22:* 635–643, 2002.
43. Jiang, W., Zhu, Z., Bhatia, N., Agarwal, R., and Thompson, H. J. Mechanisms of energy restriction: effects of corticosterone on cell growth, cell cycle machinery, and apoptosis. Cancer Res., *62:* 5280–5287, 2002.
44. Garvy, B. A., King, L. E., Telford, W. G., Morford, L. A., and Fraker, P. J. Chronic elevation of plasma corticosterone causes reductions in the number of cycling cells of the B lineage in murine bone marrow and induces apoptosis. Immunology, *80:* 587–592, 1993.
45. Garvy, B. A., Telford, W. G., King, L. E., and Fraker, P. J. Glucocorticoids and irradiation-induced apoptosis in normal murine bone marrow B-lineage lymphocytes as determined by flow cytometry. Immunology, *79:* 270–277, 1993.
46. Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science (Wash. DC), *288:* 874–877, 2000.
47. Greenberg, A. K., Hu, J., Basu, S., Hay, J., Reibman, J., Yie, T., Tchou-Wong, K. M., Rom, W. N., and Lee, T. C. Glucocorticoids inhibit lung cancer cell growth through both the extracellular signal-related kinase pathway and cell cycle regulators. Am. J. Respir. Cell Mol. Biol., *27:* 320–328, 2002.