

# Oral Consumption of Pomegranate Fruit Extract Inhibits Growth and Progression of Primary Lung Tumors in Mice

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## Abstract

To develop novel mechanism-based preventive approaches for lung cancer, we examined the effect of oral consumption of a human achievable dose of pomegranate fruit extract (PFE) on growth, progression, angiogenesis, and signaling pathways in two mouse lung tumor protocols. Benzo(a)pyrene [B(a)P] and *N*-nitroso-tris-chloroethylurea (NTCU) were used to induce lung tumors, and PFE was given in drinking water to A/J mice. Lung tumor yield was examined on the 84th day and 140 days after B(a)P dosing and 240 days after NTCU treatment. Mice treated with PFE and exposed to B(a)P and NTCU had statistically significant lower lung tumor multiplicities than mice treated with carcinogens only. Tumor reduction was 53.9% and 61.6% in the B(a)P + PFE group at 84 and 140 days, respectively, compared with the B(a)P group. The NTCU + PFE group had 65.9% tumor reduction compared with the NTCU group at 240 days. Immunoblot analysis and immunohistochemistry were used to determine effect on cell survival pathways and markers of cellular proliferation and angiogenesis. PFE treatment caused inhibition of (a) activation of nuclear factor- $\kappa$ B and I $\kappa$ B $\alpha$  kinase, (b) degradation and phosphorylation of I $\kappa$ B $\alpha$ , (c) phosphorylation of mitogen-activated protein kinases (extracellular signal-regulated kinase 1/2, c-Jun NH<sub>2</sub>-terminal kinase 1/2, and p38), (d) phosphatidylinositol 3-kinase (p85 and p110), (e) phosphorylation of Akt at Thr<sup>308</sup>, (f) activation of mammalian target of rapamycin signaling, (g) phosphorylation of c-met, and (h) markers of cell proliferation (Ki-67 and proliferating cell nuclear antigen) and angiogenesis (inducible nitric oxide synthase, CD31, and vascular endothelial growth factor) in lungs of B(a)P- and NTCU-treated mice. Thus, our data show that PFE significantly inhibits lung tumorigenesis in A/J mice and merits investigation as a chemopreventive agent for human lung cancer. [Cancer Res 2007;67(7):3475–82]

## Introduction

Lung cancer has increased in prevalence at alarming rates since the last decade, particularly because of the augmented trend in smoking, so that it is now the most common cause of cancer death in the world, representing 28% of all cancer deaths. This cancer has proven difficult to control with conventional therapeutic and surgical approaches, and the prognosis is poor with an overall 5-year survival rate of 10% to 14% in the United States (1). The fact that lung cancer is a conglomerate of diseases that elicit symptoms

at a relatively late stage is considered to be the main reason for this lack of progress. Thus, in most cases, the initial diagnosis is made when the disease has significantly advanced and is beyond the stage of current treatment options. The use of naturally occurring or synthetic agents to prevent, inhibit, or reverse lung carcinogenesis would therefore greatly benefit public health.

The pomegranate (*Punica granatum L.*) fruit has been used for centuries in ancient cultures for its medicinal purposes. For a long time, pomegranate fruit is widely consumed fresh and more recently in beverage form as juice. Pomegranate juice has been shown to suppress inflammatory cell signaling proteins in the HT-29 human colon cancer cells (2). In a murine mammary gland organ culture, fermented pomegranate juice polyphenols caused inhibition of chemically induced cancerous lesion formation (3). Pomegranate juice possesses potent antioxidant activity that results in marked protection of nitric oxide (NO) against oxidative destruction, thereby resulting in augmentation of the biological actions of NO (4). Pomegranate juice was also found to cause the up-regulation of the expression of endothelial NO synthase, which was down-regulated by oxidized low-density lipoprotein in human coronary endothelial cells (5). We extracted edible seeds of pomegranate fruit with acetone, hereafter referred to as pomegranate fruit extract (PFE). Based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, PFE was found to contain anthocyanins (such as delphinidin, cyanidin, and pelargonidin) and several hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallagic, and ellagic acid esters of glucose), which account for 92% of the antioxidant activity of the whole fruit (6). We have shown that PFE possesses remarkable antitumor-promoting effects in mouse skin (7) and antiproliferative and proapoptotic effects in prostate cancer (8). PFE was also found to protect against the adverse effects of UVB and UVA radiation by inhibiting cellular pathways in normal human epidermal keratinocytes (9, 10). We have recently reported that PFE inhibits prosurvival signaling pathways in human lung carcinoma A549 cells and inhibits tumor growth in athymic nude mice (11). Recently, the first clinical trial to determine the effects of pomegranate juice consumption on prostate-specific antigen (PSA) progression in men with rising PSA level following primary therapy was reported. Mean PSA doubling time significantly increased on treatment with pomegranate juice in men, and there was decrease in cell proliferation and increase in apoptosis in prostate cancer cells (12).

We hypothesized that PFE may be a useful chemopreventive agent for lung cancer. To test this hypothesis, we tested the effect of oral consumption of PFE against mouse models of lung tumorigenesis. We further defined the mechanistic basis of the observed chemopreventive effects. We found that PFE treatment to A/J mice exposed to benzo(a)pyrene [B(a)P] and *N*-nitroso-tris-chloroethylurea (NTCU) resulted in significant inhibition of lung tumor incidence and tumor multiplicity and inhibition of mitogen-activated protein

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kinase (MAPK), nuclear factor- $\kappa$ B (NF- $\kappa$ B), phosphatidylinositol 3-kinase (PI3K)/Akt, c-met, mammalian target of rapamycin (mTOR) signaling, markers of cellular proliferation, and angiogenesis. These data provide molecular insight into the chemopreventive effects of PFE against lung cancer by blocking signaling pathways, which are prerequisite for the development and progression of lung tumorigenesis.

## Materials and Methods

**Materials.** Extracellular signal-regulated kinase (ERK) 1/2 (phosphorylated p44/42<sup>Thr202/Tyr204</sup>), c-Jun NH<sub>2</sub>-terminal kinase (JNK) 1/2 (phosphorylated p54/46<sup>Thr183/Tyr185</sup>), p38 (phosphorylated p38<sup>Thr180/Tyr204</sup>), I $\kappa$ B $\alpha$ , phosphorylated I $\kappa$ B $\alpha$ , mTOR (phosphorylated mTOR<sup>Ser2448</sup>), eukaryotic initiation factor (eIF) 4E-binding protein 1 (4EBP1; phosphorylated 4EBP1<sup>Ser65</sup>), eIF4E (phosphorylated eIF4E<sup>Ser209</sup>), 40S ribosomal protein S6 kinase (p70S6K; phosphorylated p70S6K), and AMP-activated protein kinase (AMPK)  $\alpha$  (phosphorylated AMPK $\alpha$ <sup>Thr172</sup>) antibodies were obtained from Cell Signaling Technology (Beverly, MA). The monoclonal and polyclonal antibodies (IKK $\alpha$ ), Ki-67, proliferating cell nuclear antigen (PCNA), CD31, and vascular endothelial growth factor (VEGF) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). NF- $\kappa$ B/p65, Akt (phosphorylated Akt<sup>Thr308</sup>), PI3K (p85 and p110), c-met (phosphorylated c-met<sup>Tyr1234/Tyr1235</sup>), and inducible NO synthase (iNOS) were procured from Upstate (Lake Placid, NY). Anti-mouse and anti-rabbit secondary antibody horseradish peroxidase (HRP) conjugate was from Amersham Life Science, Inc. (Arlington Heights, IL). LightShift chemiluminescent bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). Novex precast Tris-glycine gels were obtained from Invitrogen (Carlsbad, CA).

**Preparation of PFE.** As described recently, fresh fruit of pomegranate was peeled and its edible portion (seed coat and juice) was squeezed in 70% acetone-30% distilled water (1:20, w/v). The red extract was filtered through filter paper (Whatman no.1). The filtrate was condensed and freeze dried. The freeze-dried extract was stored at 4°C. In our recent publication, we have analyzed PFE using a novel technique of MALDI-TOF MS. The PFE preparation used in this study was found to contain six anthocyanins (pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside, pelargonidin 3,5-diglucoside, cyanidin 3,5-diglucoside, and delphinidin 3,5-diglucoside) and various ellagitannins and hydrolyzable tannins (7).

**Animals.** Female strain A/J mice (The Jackson Laboratory, Bar Harbor, ME) of 7 weeks of age were used in both tumor bioassays. Throughout the experimental protocol, the mice were maintained at standard conditions under 12-h light/12-h dark cycle and fed AIN-76A diet and water *ad libitum*.

**B(a)P tumor study.** B(a)P is one of the most ubiquitous environmental polycyclic aromatic hydrocarbons known to be present in automobile exhaust and cigarette smoke, and it induces lung tumors in mice (13). Forty-eight A/J mice at 7 weeks of age were randomly distributed into four groups containing 12 mice in each group. The mice in group 1 received normal drinking water and were given the vehicle only (cottonseed oil) and served as control. The animals of groups 2 and 4 received the same drinking water supplemented with 0.2% PFE (w/v) until the termination of the experiment at day 140. The water bottles were changed every other day. The dose of PFE (0.2% PFE, w/v) selected for feeding mice is based on the assumption that a typical healthy individual (70 kg) may be persuaded to drink 500 mL of pomegranate juice extracted from two fruits. One week after treatment with PFE, mice in groups 3 and 4 were gavaged with a single dose of 20  $\mu$ mol B(a)P in 0.2 mL of cottonseed oil (Fig. 1A). At 84th day after B(a)P treatment, six mice from each group were sacrificed to quantify lung adenomas, and the lobes of lungs were placed in 10% phosphate-buffered formalin for immunohistochemical and Western blot analysis. At 140th day after B(a)P treatment, the remaining mice were sacrificed and the number of lung adenomas was recorded. We observed that no mice died prematurely or had to be sacrificed ahead of the proposed termination time.

**NTCU tumor study.** Although there are several established models for lung adenoma and adenocarcinomas, there is no well-established mouse model for lung squamous cell carcinoma (SCC). NTCU was used previously

to induce preneoplastic and neoplastic lesions, including lung SCC (14). Forty-eight A/J mice were randomly distributed into four groups containing 12 mice in each group. Forty-eight hours before initial treatment, the dorsal skin of the mice was shaved. Mice in group 1 received normal drinking water and treated with 25  $\mu$ L acetone, which is the solvent used for NTCU and served as control. Mice in groups 2 and 4 received the same drinking water supplemented with 0.2% PFE (w/v) until the termination of the experiment, and the water bottles were changed every other day. One week after treatment with PFE, mice in groups 3 and 4 were treated topically with 25  $\mu$ L of 4 mmol/L NTCU (in acetone), twice weekly, with a 3-day interval (Fig. 1C). The animals were sacrificed 240 days after the initial treatment with NTCU. Other details were exactly the same as that described for B(a)P tumor study.

**Preparation of cytosolic and nuclear lysates.** The lung tissue samples were homogenized in ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), pH 7.4] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA). The homogenate was then centrifuged at 14,000  $\times$  g for 25 min at 4°C, and the supernatant (whole-cell lysate) was collected, aliquoted, and stored at -80°C. For the preparation of cytosolic and nuclear lysates, the lung tissue samples were incubated in 0.4 mL ice-cold lysis buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF] with freshly added protease inhibitor cocktail for 15 min, after which 12.5  $\mu$ L of 10% NP40 were added and the contents were mixed on a vortex and then centrifuged for 1 min (14,000  $\times$  g) at 4°C. The supernatant was saved as cytosolic lysate and stored at -80°C. The nuclear pellet was resuspended in 50  $\mu$ L of ice-cold nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF] with freshly added protease inhibitor cocktail for 30 min with intermittent mixing. The tubes were centrifuged for 5 min (14,000  $\times$  g) at 4°C, and the supernatant (nuclear extract) was stored at -80°C. The protein concentration was determined by the BCA protein assay kit.

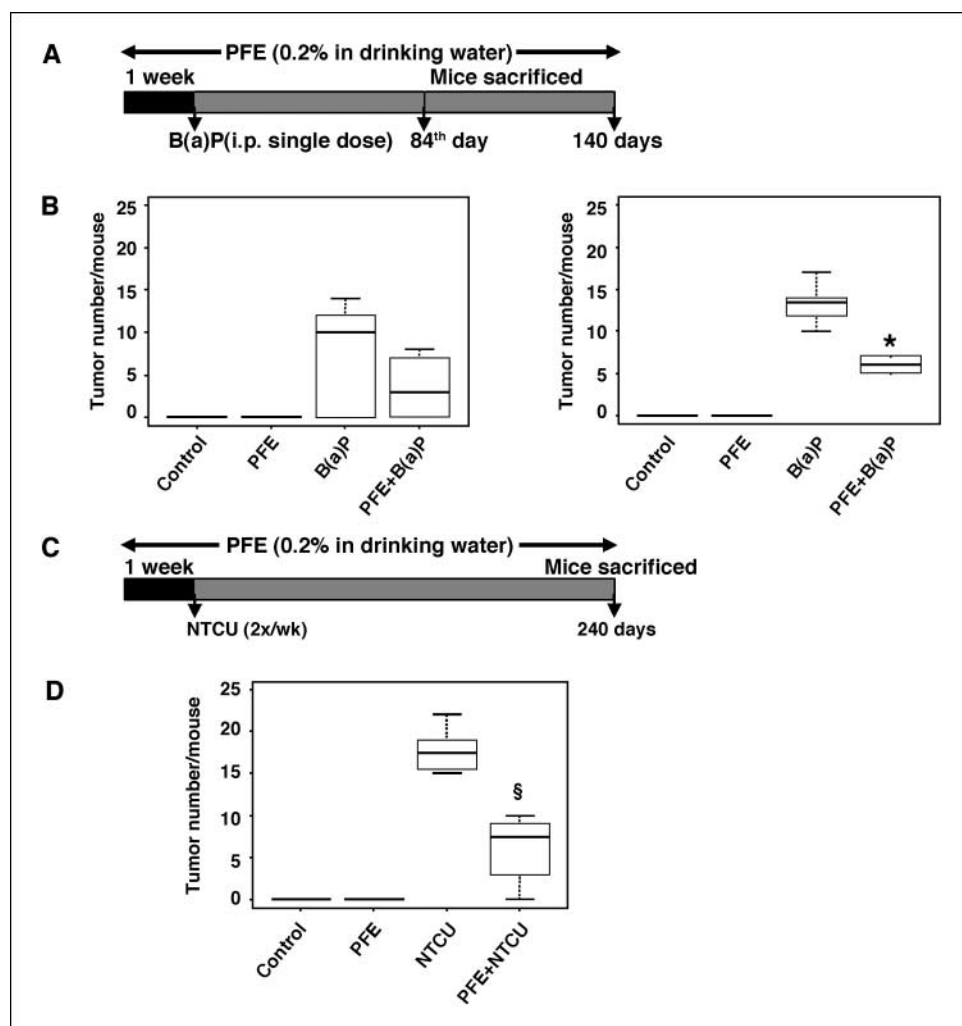
**Western blot analysis.** For immunoblotting, 30 to 50  $\mu$ g of protein were resolved over 8% to 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer [5% nonfat dry milk/1% Tween 20 in 20 mmol/L TBS (pH 7.6)] for 1 h at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for 1.5 h to overnight at 4°C followed by incubation with anti-mouse or anti-rabbit secondary antibody HRP conjugate, and detected by chemiluminescence and autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY).

**Immunohistochemical analysis.** Sections (5  $\mu$ m thick) were cut from paraffin-embedded lung tissues. Immunostaining was done using specific antibodies with appropriate dilutions and replaced with either normal host serum or block for negative controls followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counterstained with a weak solution of hematoxylin stain. The stained slides were dehydrated and mounted in permount and visualized on a Zeiss-Axiophot DM HT microscope (Zeiss-Axiophot, Jena, Germany). Images were captured with an attached camera linked to a computer.

**Statistical analysis.** Tumor incidence and tumor multiplicity data are summarized using descriptive statistics, such as proportion and mean along with 95% confidence intervals (95% CI), respectively. The observed differences in the tumor multiplicity, Ki-67, PCNA, CD31, iNOS, and VEGF data were tested for statistical significance using Student's *t* test. All statistical tests were two sided, and *P* < 0.05 was considered statistically significant. Statistical analysis was done and graphs were produced using the R language.<sup>3</sup>

<sup>3</sup> <http://www.r-project.org>

**Figure 1.** Effect of oral consumption of PFE on B(a)P- and NTCU-induced lung tumor multiplicity in A/J mice. **A**, treatment protocol for B(a)P tumor bioassay. Seven-week-old mice were given PFE (0.2%, w/v) in drinking water for 1 wk and then gavaged with a single dose of 20  $\mu$ mol B(a)P in 0.2 mL of cottonseed oil. PFE was continued in drinking water until the termination of the experiment. Six mice from each group were sacrificed on the 84th day and the remaining were euthanized after 140 d after B(a)P treatment when the experiment was terminated. **B**, lung tumor multiplicity at 84th day (*left*) and 140 d (*right*) after B(a)P treatment. Details are provided in Materials and Methods. \*,  $P < 0.001$ , B(a)P versus B(a)P + PFE group. **C**, treatment protocol for NTCU tumor bioassay. Seven-week-old mice were given PFE (0.2%, w/v) in drinking water for 1 wk and then treated topically with 25  $\mu$ L of 4 mmol/L NTCU (in acetone) twice weekly. PFE was continued in drinking water until the termination of the experiment. The mice were sacrificed 240 d after NTCU treatment. **D**, lung tumor multiplicity at 240 d after NTCU treatment. Twelve mice from each group were sacrificed after 240 d when the experiment was terminated. Details are provided in Materials and Methods. The *boxes* cover the data from the 25th to 75th percentiles; the *centerlines* represent the median; the *whiskers* extend to the minimum and maximum values within 1.5 times the length of the *box* (interquartile range), with more extreme values plotted as separate points; and the widths of a box are proportional to the square root of the sample size. §,  $P < 0.001$ , NTCU versus NTCU + PFE group.



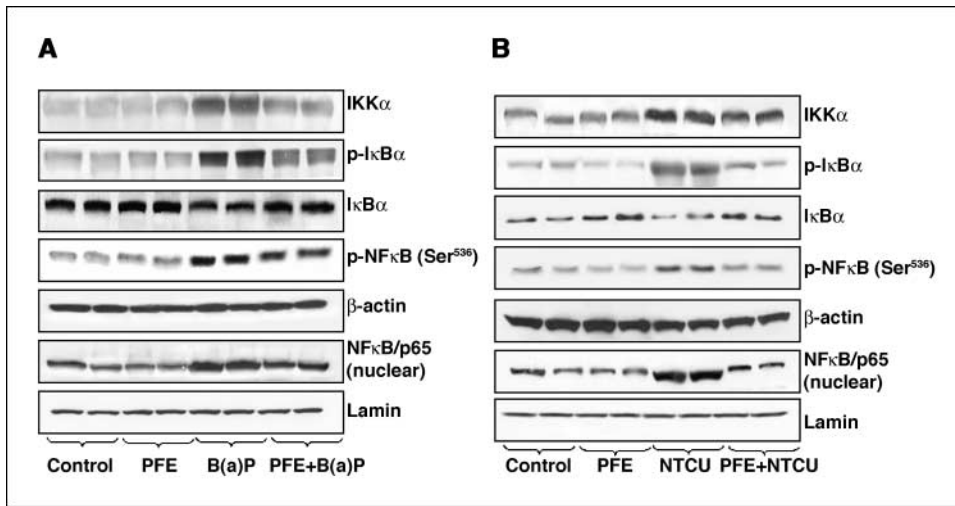
## Results

**Inhibitory effect of PFE on lung tumor multiplicity in A/J mice.** In the first experiment, we determined the effect of oral consumption of PFE on B(a)P-induced lung tumorigenesis in A/J mice (Fig. 1A). In B(a)P tumor study at interim sacrifice on the 84th day after B(a)P treatment, mice treated with B(a)P and given PFE (0.2% in drinking water) had statistically significant lower lung tumor multiplicities (i.e., mean number of tumors/mouse) than B(a)P-treated mice [7.7 tumors/mouse in the B(a)P group versus 3.5 tumors/mouse in the B(a)P + PFE group; difference, 4.2 tumors/mouse; 95% CI, -2.6 to 11.0 tumors/mouse;  $P = 0.2$ ], corresponding to a 53.9% of tumor reduction (Fig. 1B, *left*). At 140 days after B(a)P treatment, there was 61.6% of tumor reduction in mice treated with B(a)P and given PFE [13.3 tumors/mouse in the B(a)P group versus 5.2 tumors/mouse in the B(a)P + PFE group; difference, 8.1 tumors/mouse; 95% CI, 5–11.4 tumors/mouse;  $P < 0.001$ ; Fig. 1B, *right*]. Mice in groups 1 (control) and 2 (PFE treated) did not develop any tumors at any of the time point.

We next determined the effect of oral consumption of PFE on second bioassay of lung tumorigenesis induced by NTCU in A/J mice (Fig. 1C). At 240 days after NTCU treatment, NTCU-treated mice given PFE had statistically significant lower lung tumor

multiplicities than mice treated with NTCU (17.7 tumors/mouse in the NTCU group versus 6.1 tumors/mouse in the NTCU + PFE group; difference, 11.6 tumors/mouse; 95% CI, 8.9–14.3 tumors/mouse;  $P < 0.001$ ), corresponding to a 65.9% of tumor reduction (Fig. 1D). No tumors in groups 1 (control) and 2 (PFE treated) throughout the study were observed. There was no significant difference in the body weight of mice in all treatment groups in both the bioassays.

**Inhibitory effect of PFE on B(a)P- and NTCU-mediated activation of NF- $\kappa$ B and IKK $\alpha$  and phosphorylation and degradation of I $\kappa$ B $\alpha$  in A/J mice.** NF- $\kappa$ B is a ubiquitous nuclear transcription factor that plays a major regulatory role in carcinogenesis. It resides in the inactive state in the cytoplasm as a heterotrimer consisting of p50, p65, and I $\kappa$ B $\alpha$  subunits. IKK has been identified that phosphorylates serine residues in I $\kappa$ B $\alpha$ . The phosphorylation and proteolytic degradation of I $\kappa$ B $\alpha$  results in activation and nuclear translocation of NF- $\kappa$ B. To examine whether the inhibitory effect of PFE on lung tumor formation observed in the study is due to restoration of I $\kappa$ B $\alpha$ , we determined the cytoplasmic level of I $\kappa$ B $\alpha$  protein expression by immunoblot analysis. We observed that B(a)P and NTCU treatment resulted in the degradation of I $\kappa$ B $\alpha$  protein expression in lungs of A/J mice, whereas oral consumption of PFE resulted in restoration of B(a)P- and NTCU-induced degradation of I $\kappa$ B $\alpha$  protein (Fig. 2A and B).



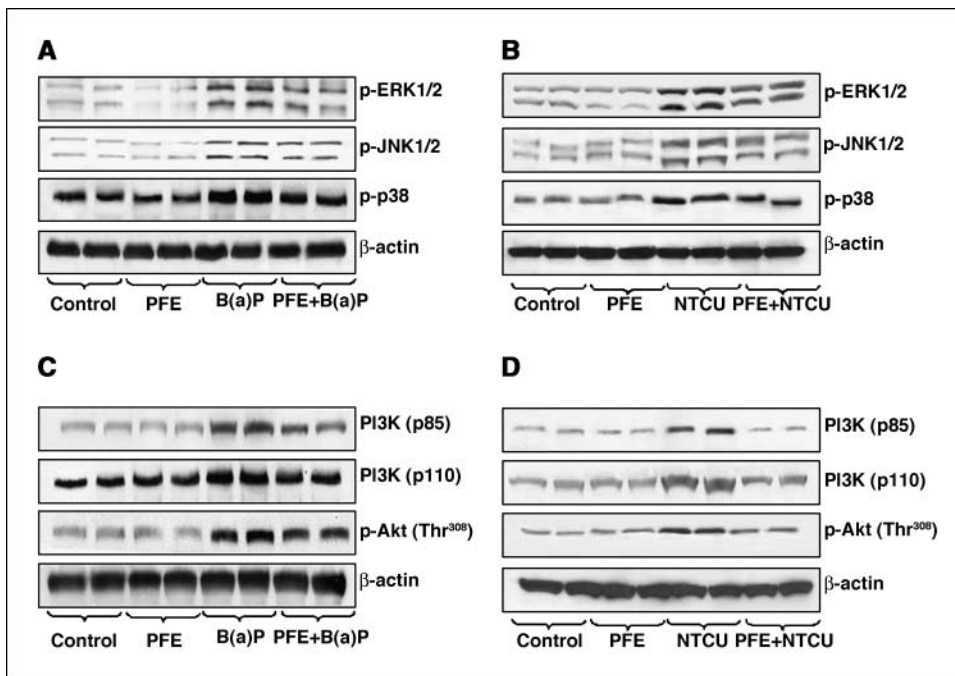
**Figure 2.** Effect of oral consumption of PFE on B(a)P- and NTCU-mediated phosphorylation and degradation of I $\kappa$ B $\alpha$  and activation of NF- $\kappa$ B and IKK $\alpha$  in lungs of A/J mice. As detailed in Materials and Methods, cytosolic and nuclear lysates of all groups were prepared from lung tissue samples from B(a)P (A) and NTCU (B) tumor studies and protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for  $\beta$ -actin. For nuclear fraction, equal loading of the protein was confirmed by reprobing it for lamin. Immunoblot analysis was conducted in all animals of each group, and only representative blots of two animals from each group are shown.

PFE-treated groups were found to inhibit B(a)P- and NTCU-induced phosphorylation of I $\kappa$ B $\alpha$  protein at Ser<sup>32</sup> (Fig. 2A and B). We also found that B(a)P and NTCU treatment caused the activation of IKK $\alpha$  protein that in turn phosphorylates and degrades the I $\kappa$ B $\alpha$  protein, resulting in the activation and nuclear translocation of p65, the functionally active subunit of NF- $\kappa$ B. PFE consumption caused inhibition of B(a)P- and NTCU-induced activation of IKK $\alpha$  and NF- $\kappa$ B/p65 (Fig. 2A and B).

**Inhibitory effect of PFE on B(a)P- and NTCU-mediated phosphorylation of MAPKs in A/J mice.** Because the activation of MAPKs is implicated in stress-induced cell death and the regulation of NF- $\kappa$ B activation, we next investigated whether the phosphorylation of MAPKs was altered by the treatment of PFE in the lungs of A/J mice. The phosphorylation of ERK1/2, JNK1/2, and p38 proteins in the whole-cell lysates was determined by immunoblot analysis (Fig. 3A and B). We found that B(a)P and NTCU treatment resulted in marked expression of the phosphor-

ylated forms of ERK1/2 (p44 and p42), JNK1/2 (p54 and p46), and p38 proteins. However, PFE (0.2% in drinking water) treatment was found to result in significant inhibition in the phosphorylated forms of all MAPK proteins (Fig. 3A and B).

**Inhibitory effect of PFE on B(a)P- and NTCU-mediated activation of PI3K and phosphorylation of Akt in A/J mice.** The PI3K/Akt pathway plays an important role in various cellular processes, including cell growth, survival, and motility. We therefore determined the effect of PFE on PI3K and phosphorylation of Akt protein expression in A/J mice. Immunoblot analysis revealed that B(a)P and NTCU treatment caused a significant increase in the expression of both p85 as well as p110 subunits of PI3K and increased phosphorylation of Akt at Thr<sup>308</sup>. PFE treatment was found to result in a significant inhibition of B(a)P- and NTCU-induced increased expression of both catalytic and regulatory subunits of PI3K and phosphorylation of Akt (Fig. 3C and D).



**Figure 3.** Effect of oral consumption of PFE on B(a)P- and NTCU-mediated phosphorylation of MAPKs and Akt and activation of PI3K in lungs of A/J mice. As detailed in Materials and Methods, whole-cell lysate of all groups was prepared from lung tissue samples from B(a)P (A and C) and NTCU (B and D) tumor studies and protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for  $\beta$ -actin. Immunoblot analysis was conducted in all animals of each group, and only representative blots of two animals from each group are shown.

**Inhibitory effect of PFE on B(a)P- and NTCU-mediated activation of mTOR signaling and phosphorylation of c-met in A/J mice.** The well-documented evidence indicating that mTOR is downstream of both PI3K and Akt led us to determine whether phosphorylation of mTOR at Ser<sup>2448</sup> was a result of PI3K/Akt activation. Treatment with B(a)P and NTCU caused increased phosphorylation of mTOR at Ser<sup>2448</sup>, whereas PFE administration resulted in inhibition of phosphorylation of mTOR (Fig. 4A and B). The mTOR integrates mitogenic signals and intracellular nutrient levels to activate 4EBP1 and p70S6K, which controls protein translation and cell cycle progression. Because mTOR activation stimulates the phosphorylation of downstream kinases, we next examined the effect of PFE on p70S6K, eIF4E, and 4EBP1 expression. B(a)P and NTCU treatment resulted in increased phosphorylation of p70S6K, eIF4E, and 4EBP1 proteins. However, PFE-treated groups showed decreased phosphorylation of the downstream targets of mTOR (Fig. 4A and B). AMPK $\alpha$  is the upstream down-regulator of mTOR. In the activated state, AMPK $\alpha$  down-regulates several anabolic enzymes and thus shuts down the ATP-consuming metabolic pathways. We therefore investigated the effect of PFE on connection between AMPK $\alpha$  and mTOR signaling. B(a)P and NTCU treatment to mice caused decreased phosphorylation of AMPK $\alpha$ , whereas treatment with PFE activated AMPK $\alpha$  as shown by the increased phosphorylation of AMPK $\alpha$  protein expression (Fig. 4A and B).

The c-met receptor tyrosine kinase and its ligand hepatocyte growth factor are involved in angiogenesis, cellular motility, growth, invasion, and differentiation. The expression of c-met has been widely investigated in several solid tumors of head and neck, esophagus, breast, kidney, and prostate. In this study, we also found that phosphorylated form of c-met was detectable with unique preferential expression in the B(a)P- and NTCU-treated lung tissues and there was decreased phosphorylation of c-met in the lung tissues of mice that received PFE (Fig. 4A and B).

**Inhibitory effect of PFE on B(a)P- and NTCU-mediated activation of cell proliferation markers in A/J mice.** Many chemopreventive trials have used various markers known to be causally linked to lung cancer as surrogate end point biomarkers, including the assessment of cell proliferation markers, such as Ki-67 and PCNA. Ki-67 is expressed in all phases of the cell cycle, except in resting cells. Because abnormal epithelial proliferation is a hallmark of tumorigenesis, the measurement of Ki-67 and PCNA in lung tissues as a surrogate end point biomarker for lung cancer

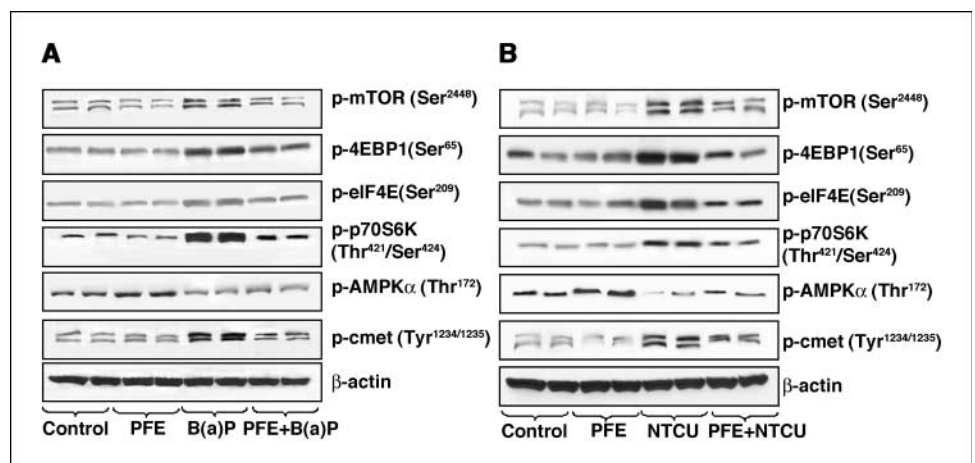
chemoprevention trials has attracted significant interest. There was marked decrease in the number of Ki-67-positive and PCNA-positive cells in the lungs of mice given PFE compared with B(a)P- and NTCU-treated mice (Fig. 5A and B). The percentage of Ki-67-positive cells was 48.3% lower in the lungs of B(a)P-treated mice that also received PFE compared with only B(a)P-treated mice and 53.3% lower in the lungs of NTCU-treated mice that received PFE compared with only NTCU-treated mice. The mean percentages of Ki-67-positive cells were 31.1 in the B(a)P-only group, 16.1 in the B(a)P + PFE group (difference, 15.0; 95% CI, 10.5–19.7;  $P < 0.001$ ), 28.2 in the NTCU-only group, and 13.1 in the NTCU + PFE group (difference, 15.1; 95% CI, 13.4–16.7;  $P < 0.001$ ; Fig. 5C, left).

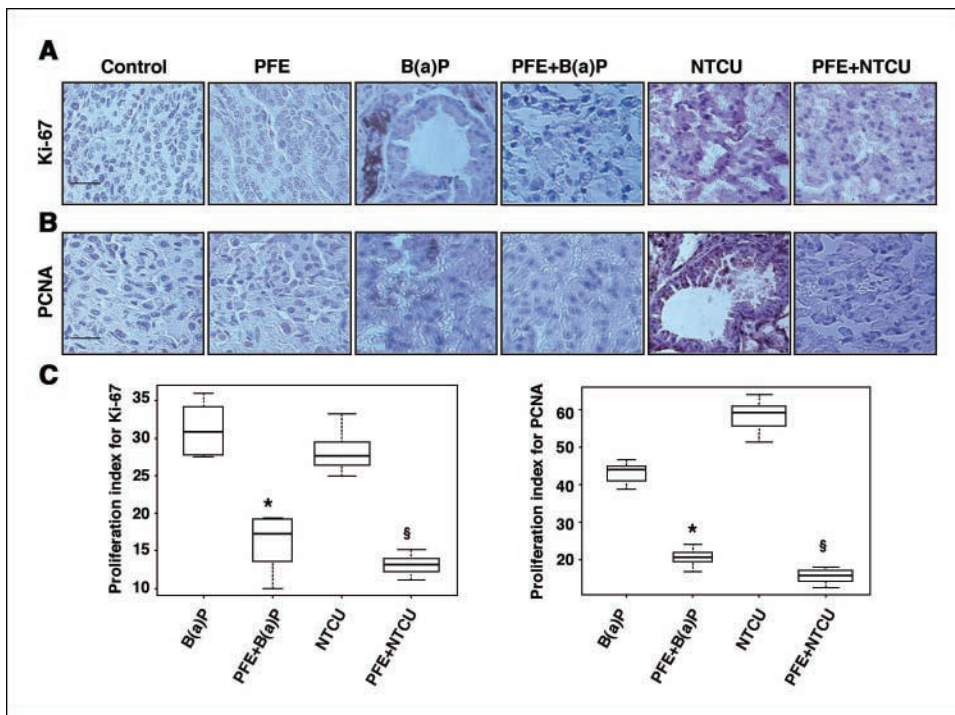
Similarly, the percentage of PCNA-positive cells was 52.7% lower in the lungs of B(a)P-treated mice that also received PFE compared with only B(a)P-treated mice and 73.5% lower in the lungs of NTCU-treated mice that received PFE compared with only NTCU-treated mice. The mean percentages of PCNA-positive cells were 43.2 in the B(a)P-only group, 20.5 in the B(a)P + PFE group (difference, 22.7; 95% CI, 19.3–26.2;  $P < 0.001$ ), 58.5 in the NTCU-only group, and 15.5 in the NTCU + PFE group (difference, 43.0; 95% CI, 40.6–45.4;  $P < 0.001$ ; Fig. 5C, right).

**Inhibitory effect of PFE on B(a)P- and NTCU-mediated activation of markers of angiogenesis in A/J mice.** Angiogenesis is essential for tumor growth and metastasis. It is strictly controlled by a highly coordinated process that is regulated by many molecules. Among them, iNOS, platelet-derived endothelial cell adhesion molecule (CD31), and VEGF are most common markers of tumor-associated angiogenesis. NO is a potentially genotoxic reactive nitrogen species formed *in vivo* by NO synthases. Tumor sections probed with an antibody to iNOS showed strong staining in lungs of B(a)P- and NTCU-treated mice. The intensity and degree of iNOS staining was markedly reduced in lung tissue sections of PFE-treated groups (Fig. 6A). PFE decreased mean iNOS immunoreactivity scores from 2.7 in the B(a)P-only group to 0.8 in the B(a)P + PFE group (difference, 1.9; 95% CI, 1.6–2.1;  $P < 0.001$ ) and from 3.2 in the NTCU-only group to 1.0 in the NTCU + PFE group (difference, 2.2; 95% CI, 2.0–2.4;  $P < 0.001$ ; Fig. 6D, left).

CD31 is a member of the immunoglobulin gene superfamily and has an important role in many endothelial cell functions, including angiogenesis, inflammation, integrin activation, and cell-cell adhesion. By immunohistochemical analysis of the lung tissue samples, we found large number of CD31-positive cells in B(a)P- and NTCU-treated mice and this was significantly lower in

**Figure 4.** Effect of oral consumption of PFE on B(a)P- and NTCU-mediated mTOR signaling and activation of c-met in lungs of A/J mice. As detailed in Materials and Methods, whole-cell lysate of all groups was prepared from lung tissue samples from B(a)P (A) and NTCU (B) tumor studies and protein was subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for  $\beta$ -actin. Immunoblot analysis was conducted in all animals of each group, and only representative blots of two animals from each group are shown.



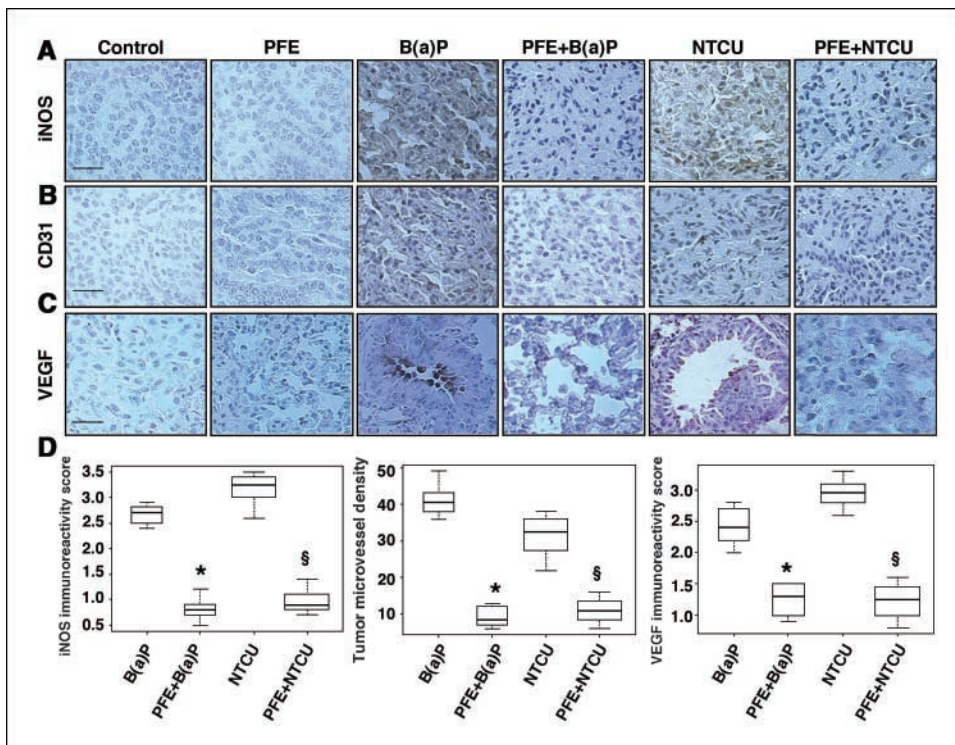


**Figure 5.** Effect of oral consumption of PFE on B(a)P- and NTCU-mediated increased expression of Ki-67 and PCNA in the lungs of A/J mice. Tumor-bearing lungs harvested from mice on the 84th day in B(a)P study ( $n = 6$  mice/group) and after 240 d in the NTCU study ( $n = 12$  mice/group) were stained using specific antibodies as detailed in Materials and Methods. Counterstaining was done with hematoxylin. Bar, 50  $\mu$ m. Representative picture from two independent immunostainings for Ki-67 (A) and PCNA (B). C, box plot, proliferation index for Ki-67 (left) and PCNA (right) with 95% CI. The boxes cover the data from the 25th to 75th percentiles; the centerlines represent the median; the whiskers extend to the minimum and maximum values within 1.5 times the length of the box (interquartile range), with more extreme values plotted as separate points; and the widths of a box are proportional to the square root of the sample size. \*,  $P < 0.001$ , B(a)P versus B(a)P + PFE group; ‡,  $P < 0.001$ , NTCU versus NTCU + PFE group.

carcinogen-treated mice that received PFE (Fig. 6B). The mice that received PFE in drinking water had 77.8% reduction in the tumor microvessel density compared with the B(a)P-only group and 65.0% reduction compared with the NTCU-only group. Mean microvessel density was 41.2 microvessels/400 $\times$  field in lungs of B(a)P-treated mice compared with 9.1 microvessels/400 $\times$  field in lungs of B(a)P + PFE-treated mice (difference, 32.1 microvessels/400 $\times$  field; 95% CI,

26.9–37.1;  $P < 0.001$ ) and 31.5 microvessels/400 $\times$  field in lungs of NTCU-treated mice compared with 11.0 microvessels/400 $\times$  field in lungs of NTCU + PFE-treated mice (difference, 20.5 microvessels/400 $\times$  field; 95% CI, 16.8–24.2;  $P < 0.001$ ; Fig. 6D, middle).

VEGF is one of the key stimuli of endothelial proliferation and migration and plays an essential role in physiologic and pathologic angiogenesis. In addition, VEGF expression has also been reported



**Figure 6.** Effect of oral consumption of PFE on B(a)P- and NTCU-mediated increased expression of iNOS, CD31, and VEGF in the lungs of A/J mice. Tumor-bearing lungs harvested from mice on the 84th day in the B(a)P study ( $n = 6$  mice/group) and after 240 d in the NTCU study ( $n = 12$  mice/group) were stained using specific antibodies as detailed in Materials and Methods. Counterstaining was done with hematoxylin. Bar, 50  $\mu$ m. Representative picture from two independent immunostainings for iNOS (A), CD31 (B), and VEGF (C). D, box plot, iNOS immunoreactivity score (left), tumor microvessel density (middle), and VEGF immunoreactivity score (right) with 95% CI. The boxes cover the data from the 25th to 75th percentiles; the centerlines represent the median; the whiskers extend to the minimum and maximum values within 1.5 times the length of the box (interquartile range), with more extreme values plotted as separate points; and the widths of a box are proportional to the square root of the sample size. iNOS and VEGF immunoreactivity score (left and right) was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), and 4+ (very strong staining). \*,  $P < 0.001$ , B(a)P versus B(a)P + PFE group; ‡,  $P < 0.001$ , NTCU versus NTCU + PFE group.

to correlate with the neoangiogenesis occurring during cancer. The lungs of mice treated with B(a)P and NTCU showed more profound expression of VEGF-positive cells than mice treated with the carcinogens and given PFE (Fig. 6C). There was a decrease in the mean VEGF immunoreactivity scores from 2.4 in the B(a)P group to 1.3 in the B(a)P + PFE group (difference, 1.1; 95% CI, 0.8–1.5;  $P < 0.001$ ) and from 3.0 in the NTCU group to 1.2 in the NTCU + PFE group (difference, 1.8; 95% CI, 1.5–1.9;  $P < 0.001$ ; Fig. 6D, right).

## Discussion

The lung cancer is increasing worldwide, and it is imperative that the development of alternative approaches, such as chemoprevention, is needed to control this epidemic in addition to continuing our efforts to strengthen the fundamental strategy of avoiding exposure to carcinogens. Dietary interventions or the use of chemopreventive agents can provide complementary approaches for cancer prevention in lowering the risk in ex-smokers and, possibly, in active smokers. Chemopreventive strategies are required to control lung cancer as it is difficult to treat with standard therapies. The continuing magnitude and severity of the lung cancer problem make it desirable to enhance smoking cessation campaigns and to make progress in early detection and chemoprevention. New specific targets for prevention are being identified with the expanded understanding of the molecular and biological mechanisms of lung cancer development. The design of successful chemoprevention trials in the future requires identification and validation of intermediate surrogate end point biomarkers that are sufficiently predictive of lung cancer development. Lessons from the treatment of advanced lung cancer and the increased understanding of important cellular signaling pathways point out that inhibiting these different regulatory cascades might prevent and even reverse lung carcinogenesis. To evaluate the effect of PFE against lung cancer in this study, we induced lung tumors by B(a)P and NTCU in A/J mice. Treatment with PFE resulted in decrease in tumor multiplicity in both B(a)P- and NTCU-treated mice. In B(a)P + PFE group, tumor reduction was 53.9% and 61.6% at 84 and 140 days, respectively, compared with B(a)P group and 65.9% in NTCU + PFE group compared with NTCU group at 240 days (Fig. 1). These data have relevance in the context of recently published study in which mean PSA doubling time significantly increased on treatment with pomegranate juice in men with a rising PSA following primary therapy (12). The results of the study provide a basis for future evaluation of the potential of PFE as chemopreventive/chemotherapeutic agent against lung cancer.

NF- $\kappa$ B transcription factor has a central role in several cellular processes, including proliferation, cell adhesion, apoptosis, inflammatory response, and regulation of the immune response (15). In the present study, we found that oral administration of PFE to A/J mice significantly inhibited B(a)P- and NTCU-induced NF- $\kappa$ B and IKK $\alpha$  activation and phosphorylation and degradation of I $\kappa$ B $\alpha$  protein (Fig. 2). The MAPKs are regulated by distinct signal transduction pathways that control many aspects of mammalian cellular physiology, including cell growth, differentiation, and apoptosis. In general, the ERK cascade is activated by growth factors and is critical for cell proliferation. Conversely, the JNK and p38 pathways are stimulated by genotoxic agents and cytokines mediating the stress response, growth arrest, and apoptosis (16). The treatment of A/J mice with PFE was found to significantly inhibit B(a)P- and NTCU-induced phosphorylation of the MAPK proteins (Fig. 3).

The PI3K/Akt signaling pathway plays a critical role in cell growth and survival. Dysregulation of this pathway has been found in a variety of cancer cells. Recently, constitutively active PI3K/Akt signaling has been firmly established as a major determinant for cell growth and survival in an array of cancers. Blocking the constitutively active PI3K/Akt signaling pathway provides a new strategy for targeted cancer therapy (17). Recently, it has been reported that tobacco carcinogens induce Akt activation and lung carcinogenesis in mice (18). Thus, inhibitors of this signaling pathway would be potential anticancer agents because survival and growth of cancer cells are dominated by constitutively active PI3K/Akt signaling. PFE treatment to A/J mice resulted in the reduction in B(a)P- and NTCU-mediated elevated expression of PI3K (p85 and p110) and phosphorylation of Akt at Thr<sup>308</sup> (Fig. 3).

The mTOR is a serine/threonine kinase, which belongs to family of phosphatidylinositol kinase-related kinase family and is involved in the regulation of a wide range of growth-related cellular functions, including transcription, translation, membrane trafficking, protein degradation, and reorganization of the actin cytoskeleton (19). The two best-characterized targets of mTOR in mammals are the ribosomal S6 kinases (S6K1 and S6K2) and 4EBP1. The activity of mTOR leads to S6K1/S6K2 phosphorylation and activation and to 4EBP1 phosphorylation and release from the cap-dependent translation initiation factor eIF4E. These two events, likely combined with other mTOR targets, lead to an increase in ribosomal biogenesis and the selective translation of specific mRNA populations. This ability to increase the protein synthesis capacity of the cell is responsible, at least in part, for the ability of the mTOR proteins to drive cell growth and proliferation and their implication in lung cancer progression (20). Overexpression of eIF4E leads to cell transformation or disordered growth (21). It has been reported that activation of either PI3K or Akt, both upstream of mTOR, is sufficient to induce the phosphorylation of both 4EBP1 and p70S6K through mTOR (22), which we have also noted in our B(a)P- and NTCU-treated samples. There is also ample evidence that treatment of activated PI3K- or Akt-expressing cells with rapamycin blocks the phosphorylation of p70S6K and 4EBP1, suggesting that mTOR is required for these activities (23). PFE-treated mice showed decreased phosphorylation of mTOR protein expression and its downstream targets, suggesting the effect of PFE on mTOR signaling (Fig. 4). AMPK $\alpha$  activation strongly suppresses cell proliferation in nonmalignant cells as well as in tumor cells. These actions of AMPK $\alpha$  seem to be mediated through multiple mechanisms, including regulation of the cell cycle and inhibition of protein synthesis. Several groups have reported that activation of AMPK $\alpha$  suppresses mTOR signaling by growth factors and amino acids (24). PFE supplementation resulted in the increased phosphorylation of the AMPK $\alpha$  protein, whereas B(a)P and NTCU treatment resulted in its decreased phosphorylation (Fig. 4). c-met is a receptor tyrosine kinase, which stimulates the invasive growth of carcinoma cells. It has been reported that c-met is overexpressed, activated, and sometimes mutated in non-small cell lung cancer cell lines and tumor tissues. It is an attractive potential target for novel therapeutic inhibition in human cancers (25). We found that lungs of B(a)P- and NTCU-treated mice showed increased phosphorylation of c-met and there was decreased phosphorylation in lungs of mice treated with PFE (Fig. 4).

Ki-67 and PCNA are well-known markers of cellular proliferation. Moreover, Ki-67 expression has been reported to reflect the level of tobacco-induced damage throughout the lung. These

characteristics suggest that Ki-67 may have added value, beyond that of histopathology, in predicating lung tumor risk and as a surrogate end point biomarker for chemopreventive efficacy. PCNA is a cofactor of DNA polymerase and is required for DNA replication and DNA nucleotide excision repair. The apparent increase in PCNA immunostaining-positive cells is a general feature of multistep carcinogenesis (26). We observed that there was markedly less expression of Ki-67 and PCNA in lungs of mice treated with PFE than in B(a)P- and NTCU-treated mice (Fig. 5). iNOS is an enzyme responsible for the production of NO. It is now widely accepted that iNOS is expressed in response to several stresses, including inflammatory cytokines, bacterial endotoxin, and cigarette smoking (27). The link between cigarette smoking and the alteration of iNOS is particularly relevant to the pathogenesis of lung cancer, as cigarette smoking is one of primary risk factors that cause lung cancer. The expression of iNOS was significantly increased in the B(a)P- and NTCU-treated mice than in PFE-treated mice (Fig. 6). CD31 has been known to be a reliable marker for endothelial differentiation. This is important in revealing possible correlation of tumor angiogenesis with metastatic behavior, prognosis, or angiogenic factor over-expression (28). PFE-treated groups displayed fewer CD31-stained blood capillaries compared with B(a)P- and NTCU-treated groups, which showed higher CD31-positive cells as visualized by brown color staining (Fig. 6). VEGF is the major regulator of tumor-associated angiogenesis in lung adenocarcinoma, which can

promote tumor growth and metastasis. Inhibition of VEGF reduces angiogenesis and tumor growth *in vivo* (29). The present study shows that VEGF was extensively expressed in the lungs of B(a)P- and NTCU-treated mice and this expression was markedly decreased in PFE-treated mice, suggesting that down-regulation of VEGF is a possible mechanism by which PFE exerts its antiangiogenic action (Fig. 6).

In conclusion, the results from this study clearly show that oral consumption of PFE effectively inhibited lung tumor multiplicity and incidence induced by B(a)P and NTCU. PFE treatment also down-regulated the activation of (a) MAPKs, (b) NF- $\kappa$ B, (c) PI3K/Akt, (d) mTOR signaling, and (e) c-met. PFE also caused reduced expressions of markers of cell proliferation and angiogenesis in lungs of B(a)P- and NTCU-treated mice. Furthermore, the present study suggests that PFE inhibits lung tumorigenesis by targeting multiple signaling pathways and associated events and, therefore, strongly supports its development as chemopreventive agent against human lung cancer.

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