

## $\beta$ -Cryptoxanthin Restores Nicotine-Reduced Lung SIRT1 to Normal Levels and Inhibits Nicotine-Promoted Lung Tumorigenesis and Emphysema in A/J Mice

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### Abstract

Nicotine, a large constituent of cigarette smoke, is associated with an increased risk of lung cancer, but the data supporting this relationship are inconsistent. Here, we found that nicotine treatment not only induced emphysema but also increased both lung tumor multiplicity and volume in 4-nitrosamino-1-(3-pyridyl)-1-butanone (NNK)-initiated lung cancer in A/J mice. This tumor-promoting effect of nicotine was accompanied by significant reductions in survival probability and lung Sirtuin 1 (SIRT1) expression, which has been proposed as a tumor suppressor. The decreased level of SIRT1 was associated with increased levels of AKT phosphorylation and interleukin (*il*)-6 mRNA but decreased tumor suppressor *p53* and retinoic acid receptor (*RAR*)- $\beta$  mRNA levels in the lungs. Using this mouse model, we then determined whether  $\beta$ -cryptoxanthin (BCX), a xanthophyll that is strongly associated with a reduced risk of lung cancer in several cohort studies, can inhibit nicotine-induced emphysema and lung tumorigenesis. We found that BCX supplementation at two different doses was associated with reductions of the nicotine-promoted lung tumor multiplicity and volume, as well as emphysema in mice treated with both NNK and nicotine. Moreover, BCX supplementation restored the nicotine-suppressed expression of lung SIRT1, *p53*, and *RAR*- $\beta$  to that of the control group, increased survival probability, and decreased the levels of lung *il*-6 mRNA and phosphorylation of AKT. The present study indicates that BCX is a preventive agent against emphysema and lung cancer with SIRT1 as a potential target. In addition, our study establishes a relevant animal lung cancer model for studying tumor growth within emphysematous microenvironments. *Cancer Prev Res*; 6(4); 309–20. ©2012 AACR.

### Introduction

Despite anti-smoking campaigns and efforts toward prevention and treatment, lung cancer accounts for 14% of all new cancers. Approximately 80% to 90% of lung cancers occur in smokers (1). Smoking is the leading cause of chronic obstructive pulmonary disease (COPD), which exists in 2 forms: chronic bronchitis and emphysema (2). Emphysema, a permanent enlargement of the lung airspaces that is accompanied by alveolar wall

destruction, is associated with a 3- to 4-fold increased lung cancer risk (3).

More than 60 carcinogens present in cigarette smoke are accountable for lung carcinogenesis (1, 4). Nicotine, a noncarcinogenic constituent of tobacco and cigarette smoke, is responsible for smoking addiction (5). Smokers who switch from smoking to using smokeless tobacco products containing nicotine have a higher risk of lung cancer than those who stopped smoking completely (5). Previous animal studies showed that intraperitoneal (i.p.) and oral administrations of nicotine promotes lung tumorigenesis in female A/J mice (6) and in the mouse Lewis lung tumor models (7), respectively. However, 2 studies reported that nicotine in drinking water does not promote lung tumorigenesis in female A/J mice (8) or the AB6F1 mouse models (9). The discrepancy may be due to strain variability and gender as well as dosage, metabolism, and route of administration of nicotine. Although emphysema develops in the offspring of rats exposed to nicotine during gestation and lactation (10), no study had addressed lung tumor development within emphysematous microenvironments.

Sirtuin 1 (SIRT1), the mammalian ortholog of yeast *sir2*, is a NAD<sup>+</sup>-dependent deacetylase that has been implicated in various biologic processes such as metabolism,

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inflammation, immune function, and apoptosis (11). SIRT1 displaces acetyl groups from histones and proteins (e.g., forkhead box class O, NF- $\kappa$ B, p300, and the DNA repair factors Ku70; ref. 11). Smoke exposure induces emphysema in heterozygous *sirt1*<sup>+/-</sup> mice (12). Moreover, SIRT1 levels are reduced in smokers and patients with COPD (13). Although modulating SIRT1 with activators is an attractive therapeutic strategy in COPD/emphysema (12), SIRT1 roles have not been explored in lung tumorigenesis, particularly during the promotion and progression phases. SIRT1 was originally identified as a tumor promoter (11). However, studies using genetically modified *sirt1* mice have consistently supported a tumor-suppressive role of SIRT1 in a wide range of cancers (11).

No dietary components have been shown to prevent the progression of emphysema and lung cancer. The beneficial effect of carotenoid-rich fruits and vegetables against lung cancer risk has been reported in epidemiologic studies. Most research has focused on the pro-vitamin A carotenoid  $\beta$ -carotene because of the association between high levels of both vitamin A and  $\beta$ -carotene and lower cancer risk. Nevertheless, intervention studies using  $\beta$ -carotene have led to disappointing results (14). Recently,  $\beta$ -cryptoxanthin (BCX), an oxygenated carotenoid (xanthophyll) with pro-vitamin A activity found at high levels in citrus fruits, pumpkins, red peppers, and papayas was identified to be the only carotenoid for which intake was inversely associated with lung cancer risk in a pooled analysis of 7 prospective cohort studies (15). This intake of BCX produced a protective association among current smokers but not among past smokers or never smokers (15). Furthermore, a meta-analysis reported that BCX intake is associated with a lower lung cancer risk in current smokers (16). We previously reported that BCX treatment reduces the growth of immortalized bronchial epithelial and non-small cell lung cancer cells, induces retinoic acid receptor (*RAR*)- $\beta$  levels, and increases the transactivation activity of the retinoic acid response element-dependent *RAR*- $\beta$  promoter (17). Recently, we showed that BCX supplementation reduced cigarette

smoke-induced lung inflammation and precancerous lesions (e.g., squamous metaplasia), as well as suppressed cigarette smoke-activated NF- $\kappa$ B and TNF- $\alpha$  protein expression in ferrets (18). However, this ferret study could not address the efficacy of BCX supplementation on lung tumor formation and promotion. Furthermore, delineating the potential mechanism(s) of BCX against lung tumorigenesis is needed.

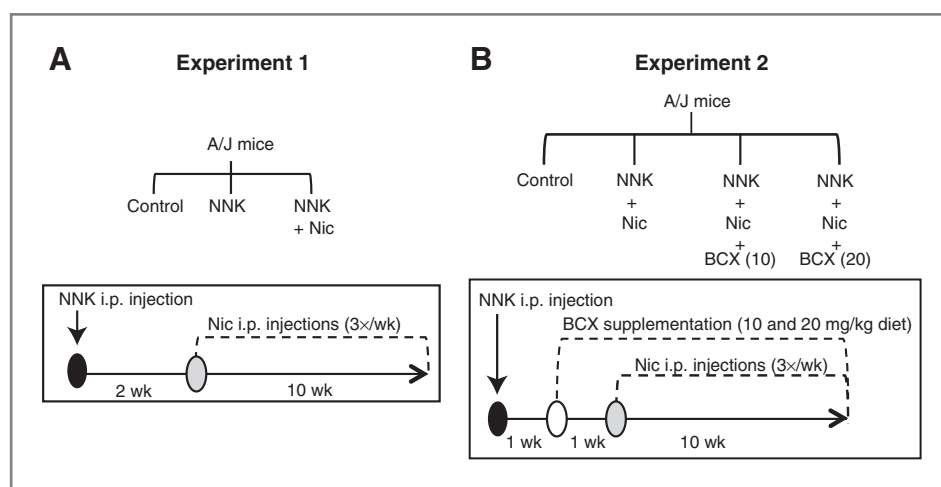
In the present study, we first investigated whether nicotine exposure altered lung SIRT1 expression and promoted lung tumorigenesis. Furthermore, we determined the effects of BCX supplementation on this nicotine-promoted tumorigenesis and emphysema.

## Materials and Methods

### Animals and experimental groups

Male A/J mice (5–6 weeks old) were purchased from the Jackson Laboratory. To investigate whether nicotine promoted both emphysema and lung tumorigenesis, we carried out experiment 1 (Fig. 1A): mice were divided into 3 groups with 16 mice per group: (i) the control group received sham injections, (ii) the 4-nitrosamino-1-(3-pyridyl)-1-butanone (NNK) group received a single intraperitoneal (i.p.) NNK injection [100 mg/kg body weight (bw)], and (iii) the NNK + Nic group received a single i.p. NNK injection (100 mg/kg bw) and i.p. nicotine injections (1 mg/kg bw) 3 times weekly for 10 weeks. No statistically significant differences were detected among the mean body weight of all groups at the beginning of the study (Supplementary Table S1).

To determine whether BCX protects against nicotine-promoted emphysema and lung tumorigenesis, we carried out experiment 2 (Fig. 1B): mice were divided into 4 groups with 16 mice per group: (i) the control group received sham injections, (ii) the NNK + Nic group received a single i.p. NNK injection (100 mg/kg bw) and i.p. nicotine injections (1 mg/kg bw) 3 times weekly for 10 weeks, (iii) NNK + Nic + BCX (10) group received a single i.p. NNK injection (100 mg/kg bw), i.p. nicotine injections (1 mg/kg bw) 3 times



**Figure 1.** Study design and experiment schemes. The study design (top) and experiment scheme (bottom) of experiment 1, which tested the effects of nicotine on promoting lung tumor and emphysema development (A), and experiment 2, which tested the effect of BCX supplementation on the promotion of lung tumor and emphysema development (B), are shown. Abbreviations: BCX (10):  $\beta$ -cryptoxanthin at 10 mg/kg diet; BCX (20):  $\beta$ -cryptoxanthin at 20 mg/kg diet; C, control; i.p.: intraperitoneal.

weekly for 10 weeks, and a diet supplemented with BCX (10 mg/kg diet), and (iv) NNK + Nic + BCX (20) group received a single i.p. NNK injection (100 mg/kg bw), i.p. nicotine injections (1 mg/kg bw) 3 times weekly for 10 weeks, and a diet supplemented with BCX (20 mg/kg diet).

The body weights of the mice were recorded weekly. After the experimental periods, the mice were terminally exsanguinated under deep isoflurane anesthesia. The studies were conducted with the approval of the Animal Care and Use Committee at the Human Nutrition Research Center on Aging at Tufts University (Boston, MA).

### Carcinogen and nicotine treatment

The tobacco-specific carcinogen NNK (>98% purity, Toronto Research Chemicals) was injected i.p. (100 mg/kg bw) once into mice to induce lung tumors, as previously described (1). Nicotine (N3876) (Sigma-Aldrich) was injected i.p. into mice 3 times weekly at 1 mg/kg bw (Fig. 1A and B). This dose of nicotine (a weekly dose of 3 mg/kg bw) corresponds to a daily dose of  $0.428 \approx 0.43$  mg/kg bw. Because a 60-kg person absorbs approximately 1 mg of nicotine (0.017 mg nicotine/kg bw) from smoking one cigarette (19), the dose of nicotine in this present animal study was equivalent to smoking 25 cigarettes per day ( $0.017$  mg nicotine/kg bw  $\times$  25 cigarettes =  $0.425 \approx 0.43$  mg nicotine/kg bw) in humans, which is the mean level of cigarette consumption in the United States (20).

### $\beta$ -Cryptoxanthin treatment

BCX (>99% purity, BASF) was in powder form and directly mixed with AIN-93M semipurified diet powder (Dyets, Inc.) at concentrations of 10 and 20 mg/kg diet (equivalent to 2 and 4 mg/kg bw daily, respectively, for a 25-g mouse that consumes 5 g daily). The absorption of carotenoids in rodents is approximately one seventh that in humans [a daily supplementation of 20 mg  $\beta$ -carotene resulted in serum concentrations of  $\beta$ -carotene of 3000  $\mu$ g/L in humans (ref. 21) and of  $\sim 416$   $\mu$ g/L (range, 134–698  $\mu$ g/L) in mice (refs. 22, 23)]. Moreover, we found that a daily supplementation of 10 mg BCX/kg diet resulted in a serum concentration of BCX of 9.9  $\mu$ g/L in A/J mice (unpublished data) and the absorption of BCX in humans is approximately 8% of what is consumed (a diet consumption of 1300  $\mu$ g BCX resulted in a serum concentration of 113  $\mu$ g/L; ref. 24). Therefore, the low dose of BCX (10 mg/kg diet) in this mouse study was equivalent to daily human consumption of 0.87 mg [ $9.9$   $\mu$ g/L  $\times 7 \times (100\%/8\%) = 866.25$   $\mu$ g = 0.87 mg], and the high dose of BCX (20 mg/kg diet) in this study was equivalent to daily human consumption of approximately 1.74 mg of BCX. These doses can be obtained from dietary citrus fruits, such as by consuming 3 to 5 raw tangerines daily (25); thus, the doses of BCX supplementation used here are within the physiologic range.

### Quantification of lung lesions

Lung tumor lesions were quantified by determining the incidence and multiplicity of the pulmonary surface tumors on the day of euthanasia as previously described (26). Lung

tumor multiplicity was used as an indicator of carcinogenicity as recommended (27). The diameter of each tumor was calculated using a caliper. The volume of each lung surface tumor was calculated on the basis of the assumption that the tumor was spherical (volume =  $4/3\pi r^3$ , where  $r$  = diameter/2; ref. 28). The upper right lungs were perfused with 10% buffered formaldehyde (Fisher Scientific) using standardized protocol by researcher blinded to the treatment group as previously described (26, 29). After the fixation for 24 hours, the lung tissues were transferred to 70% ethanol for a routine histologic processing. Hematoxylin and eosin (H&E)-stained lung sections were microscopically examined to confirm the presence of lung tumors and emphysema that was based on the histologic pattern of enlarged alveolar spaces with club-shaped septa distal to the terminal bronchioles in the H&E-stained lung sections. As previously suggested, a morphologic evaluation should be conducted for biologic studies on the pathogenesis of emphysema (30). These pathologic evaluations on lung tumors and emphysema were conducted by researchers blinded to the treatment groups.

### Quantification of emphysema

The degree of emphysema was assessed under the microscope by comparing the airspace enlargement [alveolar space area, mean linear intercept ( $L_m$ )] of the H&E-stained lung sections in a blinded manner. The  $L_m$  was measured by placing a  $0.5 \times 0.5$   $\mu$ -pixel grid over each field (10 field/mice). The  $L_m$  is calculated by dividing the total length of each line of the grid by the number of alveolar intercepts (31).

### Protein isolation and Western blotting

Whole-cell lysates of lung tissues were prepared as previously described (26). The whole-cell lysate samples were collected and either kept at  $-80^\circ\text{C}$  or used for Western blotting as previously described (26). The following antibodies were used for Western blotting: SIRT1, cyclin D1, and p50 (Santa Cruz Biotechnology, Inc.); phosphorylated AKT (Ser473) and total AKT (Cell Signaling Technology); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore). All of the antibodies were used according to the manufacturers' protocols.

### RNA extraction and quantitative real-time PCR

An RNeasy kit (Qiagen) was used to extract RNA according to the manufacturer's protocol and as previously described (26). cDNA was prepared from the RNA samples using M-MLV reverse transcriptase (Invitrogen) and an automated thermal cycler PTC-200 (MJ Research). Real-time PCR was carried out using FastStart Universal SYBR Green Master (ROX) (Roche). The relative gene expression was determined using the  $2^{-\Delta\Delta C_t}$  method. Primer sequences are listed in Supplementary Table S2.

### High-performance liquid chromatography

The livers samples for high-performance liquid chromatography (HPLC) analysis were prepared as previously

described (32). A gradient reverse-phase HPLC system consisting of a Waters 2695 separation module and a Waters 2998 photodiode array detector were used for the detection of BCX, retinol, and retinyl palmitate. Briefly, BCX, retinol, and retinyl palmitate were analyzed on a reverse-phase C18 column (4.6 × 250 mm, 5 μm; Vydac 201TP54, Grace Discovery Sciences, Inc.) with a flow rate of 1.00 mL/min and quantified relative to an internal standard by determining the peak areas against known amounts of standards.

### Statistical analyses

The mean values for each group were compared using a *t* test or a one-way ANOVA with Tukey's honestly significant difference (HSD) *post hoc* procedure applied for comparisons across multiple groups. The Statistical Analysis System (SAS version 9.2) PROC LIFETEST, a nonparametric procedure for estimating survival rates, was used for the survival analysis. Kaplan–Meier survival curves were constructed to compare the survival of mice across the treatment groups using the log-rank test. All analyses were conducted using SAS. All measurements are expressed as the mean ± SEM or as otherwise indicated. Differences were considered significant if  $P < 0.05$ .

## Results

### Nicotine decreases survival probability but not body weight

At the time of sacrifice, the mean body weight of the NNK group was lower than the control group (Supplementary Table S1). The mean body weight of the NNK + Nic group was not different than that of the NNK group. The NNK + Nic group exhibited decreased survival probability compared with the control and NNK groups (NNK + Nic group compared with control or NNK group;  $P < 0.01$ ; log-rank test). The NNK treatment alone did not alter the survival probability as compared with the control group ( $P = 0.94$ ; log-rank test; Fig. 2A).

### Nicotine promotes NNK-induced lung tumor multiplicity and volume

The mean multiplicity of spontaneous tumors (due to *Kras* mutations; ref. 33) in the control group was  $1.86 \pm 0.36$  (Fig. 2B). The mean lung tumor multiplicity of the NNK group ( $4.3 \pm 0.5$ ) was twice as that of the control group ( $P < 0.01$ ; Fig. 2B). The lung tumor volume of the NNK group ( $1.29 \pm 0.07 \text{ mm}^3$ ) was 2.5-fold higher than that of the control group ( $0.52 \pm 0.35 \text{ mm}^3$ ; Fig. 2C). Moreover, the mean lung tumor multiplicity of the NNK + Nic group ( $9.7 \pm 1.7$ ) was 2.5-fold greater than the NNK group ( $P < 0.01$ ; Fig. 2B), and the lung tumor volume of the NNK + Nic group ( $2.32 \pm 0.48 \text{ mm}^3$ ) was 79% larger than the NNK group ( $P < 0.01$ ; Fig. 2C).

### Nicotine induces emphysema development

The incidence of emphysema in the NNK + Nic group (90%) was greater than that in the control and NNK groups ( $P < 0.01$ ; Fisher's exact test). The  $L_m$  of the NNK + Nic

group was greater than the NNK group and the control group ( $P < 0.01$ ; Fig. 2D and E). The  $L_m$  for the NNK group did not differ from that of the control group ( $P = 0.13$ ; Fig. 2D and E).

The mRNA level of lung *interleukin (il)-6* mRNA in the NNK + Nic group was 3.5-fold greater than that in the NNK group ( $P < 0.01$ ; Fig. 2F). The *il-6* mRNA level in the NNK group was not different from the control group ( $P > 0.05$ ).

The level of cyclin D1 in the NNK + Nic group was decreased, by 30%, as compared with the control group ( $P < 0.05$ ; Fig. 2G). NNK treatment was not associated with a decreased cyclin D1 protein levels (NNK group compared to control group;  $P = 0.65$ ).

We observed a decreased p50 protein in the NNK + Nic group compared with control (Fig. 2H). However, this decrease was not statistically significant compared with the NNK group ( $P = 0.09$ ; Fig. 2H).

There was a 28-fold increase in the *egr1* mRNA level in the NNK + Nic group compared with the NNK or control group (Fig. 2I). Treatment of NNK did not affect the p50 protein and *egr1* mRNA levels (NNK group compared with control group;  $P > 0.05$ ; Fig. 2H and I).

### Nicotine decreases the SIRT1 protein levels in the lungs

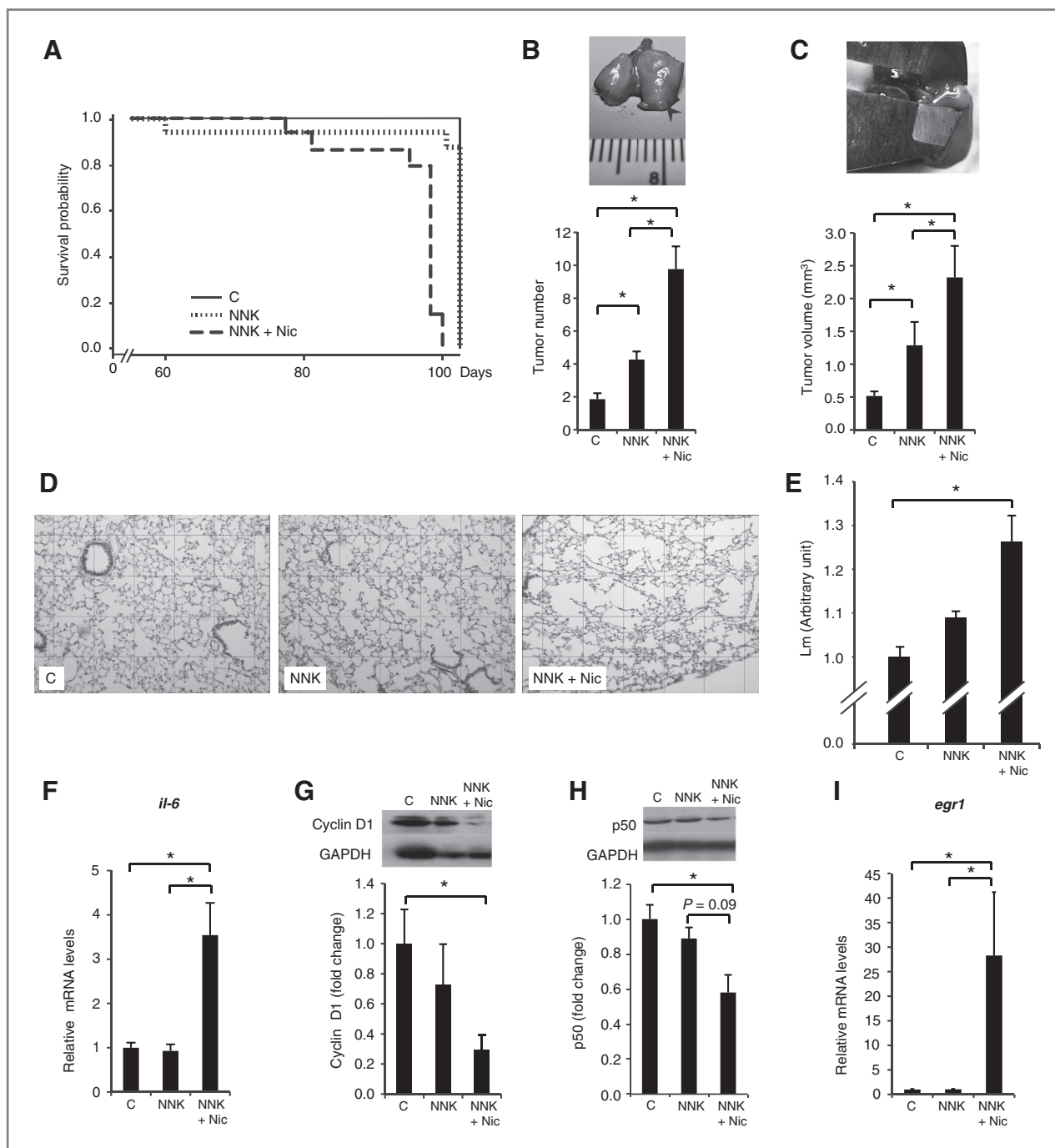
The lung levels of the SIRT1 protein in the NNK + Nic group was decreased, by 52%, compared with the NNK group ( $P < 0.01$ ), and by 56%, compared with the control group ( $P < 0.01$ ; Fig. 3A). The SIRT1 protein level in the NNK group did not differ from the control group ( $P > 0.05$ ; Fig. 3A). *Sirt1* mRNA levels in the lungs were similar across all groups (Fig. 3B).

### Nicotine enhances AKT phosphorylation and decreases the mRNA levels of both p53 and RAR-β

There was an increase in the lung AKT phosphorylation level in the NNK + Nic group compared with the control group ( $P < 0.01$ ; Fig. 3C). The lung mRNA level of *p53* was reduced 61% in the NNK + Nic group as compared with the control and NNK groups ( $P < 0.01$ ; Fig. 3D). NNK treatment did not alter the lung *p53* mRNA levels compared with the control group (Fig. 3D). A decrease (49%) in the lung *RAR-β* mRNA levels was observed in the NNK + Nic group compared with the control group ( $P < 0.01$ ; Fig. 3E). Nonetheless, the lung *RAR-β* mRNA levels in the NNK group did not differ from the control group (Fig. 3E).

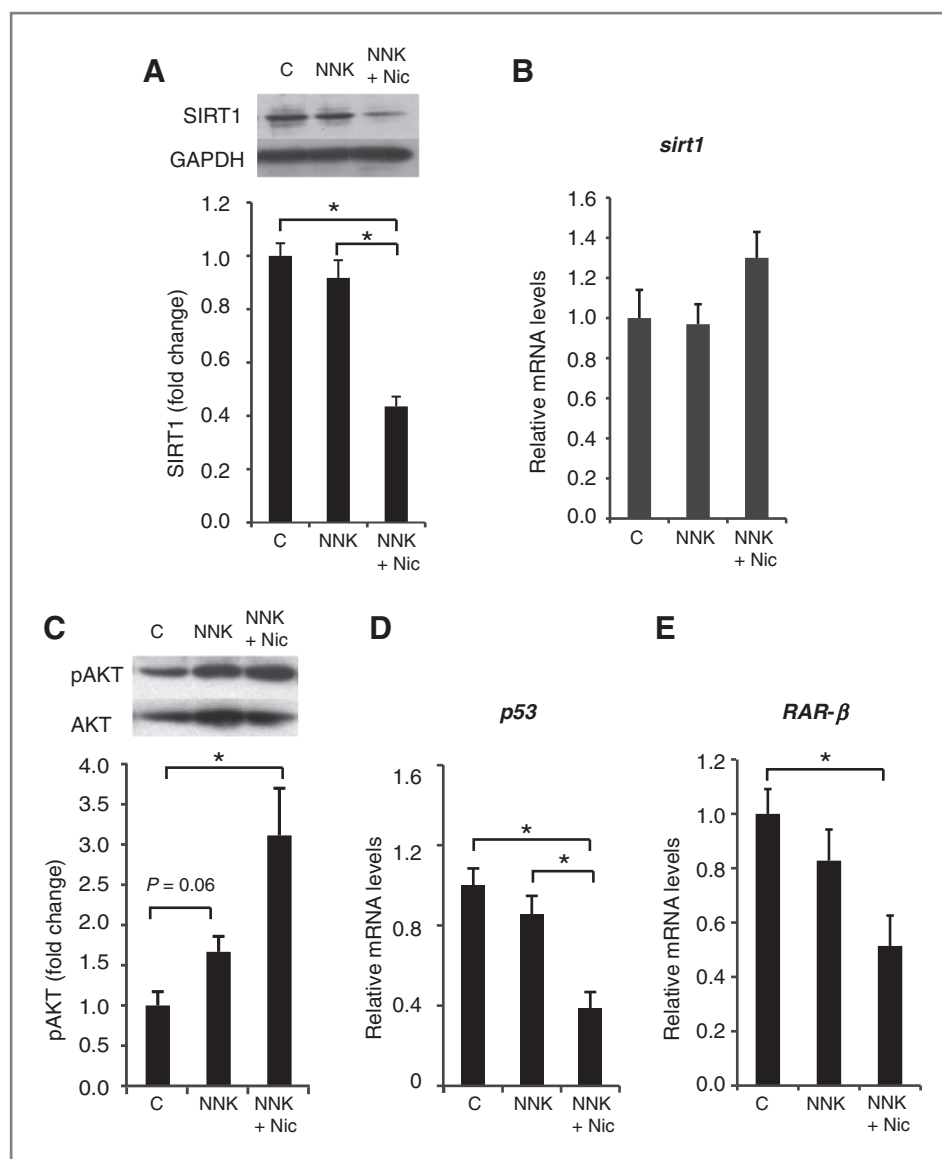
### BCX supplementation maintains body weights, increases survival probability and BCX accumulation in the liver

At the time of sacrifice, the mean body weight of the BCX-supplemented groups was not different than the control group (Supplementary Table S1). Because of limited lung samples, we examined the livers for BCX accumulation. We did not detect BCX in the livers of mice without BCX supplementation (Table 1). BCX supplementation at 10 and 20 mg/kg diet dose dependently increased the



**Figure 2.** Nicotine decreases survival probability, lung tumor multiplicity and size, and emphysema development. The survival curves for the 3 treatment groups in experiment 1 were analyzed using the SAS PROC LIFETEST. A, the results of the log-rank test for the control, NNK, and NNK + Nic groups ( $P < 0.01$ ). B, the quantification of the lung tumor multiplicity (B). Inset, lung surface tumors (arrows). C, the number of lung surface tumors and the quantification of the lung tumor volume (mm<sup>3</sup>). Inset, the measurement of the lung tumor diameter with a caliper. D, representative images of the H&E-stained slides (grid: 0.5 × 0.5 μ-pixels). E, the degree of emphysema was quantified by calculating L<sub>m</sub> using 10 fields per animal. F, the lung *il-6* mRNA levels. G, the lung cyclin D1 protein levels. Inset, a representative image of the cyclin D1 and GAPDH proteins. H, the lung p50 protein levels. Inset, a representative image of the p50 and GAPDH proteins. I, the lung *egr1* mRNA levels. The proteins were detected using Western blotting. The intensity of the bands was quantified densitometry and was normalized to GAPDH. The mRNA levels were measured by quantitative real-time PCR (qRT-PCR) and normalized to *β-actin* mRNA levels. The columns represent the mean ± SEM. \*,  $P < 0.05$ . C, control; *egr1*, early growth response 1.

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**Figure 3.** Nicotine decreases the level of SIRT1 protein and the transcription of tumor suppressor genes and increases AKT phosphorylation. A, the lung SIRT1 protein levels. Inset, a representative image of lung SIRT1 and GAPDH proteins. B, the lung *sirt1* mRNA levels. C, the quantification of AKT phosphorylation on Ser473. Inset, a representative image of pAKT and total AKT. The mRNA levels of the (D) *p53* and (E) *RAR-β* are shown. The proteins were detected using Western blotting. The intensity of the bands was quantified using densitometry and normalized to GAPDH or total AKT. The mRNA levels were measured with quantitative real-time PCR (qRT-PCR) and normalized to  $\beta$ -actin mRNA levels. The columns represent the mean  $\pm$  SEM. \*,  $P < 0.05$ . C, control.

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**Table 1.** Concentrations of BCX and retinoids in the liver after 11 weeks of BCX supplementation

| Treatment group                 | Liver, nmol/g                |                               |                              |
|---------------------------------|------------------------------|-------------------------------|------------------------------|
|                                 | BCX                          | Retinol                       | Retinyl palmitate            |
| Control                         | ND                           | 12.51 $\pm$ 1.28              | 1,240 $\pm$ 46               |
| NNK + Nic                       | ND                           | 14.20 $\pm$ 2.19              | 1,457 $\pm$ 86               |
| NNK + Nic + BCX (10 mg/kg diet) | 0.25 $\pm$ 0.03              | 13.45 $\pm$ 1.24              | 1,626 $\pm$ 47 <sup>b</sup>  |
| NNK + Nic + BCX (20 mg/kg diet) | 0.51 $\pm$ 0.04 <sup>a</sup> | 18.95 $\pm$ 1.56 <sup>b</sup> | 2,017 $\pm$ 141 <sup>c</sup> |

NOTE: Values are expressed as the mean  $\pm$  SEM.  $n = 12$ –14. The statistical analyses consisted of an ANOVA followed by *post hoc* testing using Tukey honestly significant difference (HSD) test, unless otherwise indicated.

Abbreviation: ND, not detected.

<sup>a</sup> $P < 0.05$  compared with NNK + Nic + BCX (10 mg/kg diet) using Student *t* test.

<sup>b</sup> $P < 0.05$  compared with the control.

<sup>c</sup> $P < 0.05$  compared with NNK + Nic.

concentration of liver BCX ( $P < 0.01$ ; Table 1). We observed no significant differences in the hepatic retinol concentration across the groups (Table 1). However, the retinyl ester concentration was increased in the livers of mice supplemented with the high dose of BCX as compared with the control group (Table 1).

Both doses of BCX maintained the survival probability at the level of the control group [(Fig. 4A); NNK + Nic + BCX (10) compared with control group ( $P = 0.99$ ) and NNK + Nic + BCX (20) compared with control group ( $P = 0.99$ ); log-rank tests]. The 2 doses of BCX supplementation increased the survival probability of the mice compared with the NNK + Nic group [(Fig. 4A); NNK + Nic + BCX (10) group compared with NNK + Nic group ( $P < 0.01$ ) and NNK + Nic + BCX (20) group compared with NNK + Nic group ( $P < 0.01$ ); log-rank tests].

### BCX inhibits the nicotine-promoted lung tumor multiplicity/volume and emphysema

The supplementation of BCX at 10 and 20 mg/kg diet decreased the lung tumor multiplicity by 86% and 91%, respectively, compared with the NNK + Nic group (Fig. 4B). The lung tumor volumes of the mice supplemented with 10 and 20 mg BCX/kg diet were 79% and 87% smaller, respectively, compared with the NNK + Nic group (Fig. 4C). The incidences of emphysema in the NNK + Nic + BCX (10; 17%) and NNK + Nic + BCX (20; 17%) groups were lower than in the NNK + Nic group (90%;  $P < 0.01$ ). Moreover, the  $L_m$  of the lungs of the NNK + Nic + BCX (10) and NNK + Nic + BCX (20) groups were lower than in the NNK + Nic group ( $P < 0.01$ ; Fig. 4D).

The 10 and 20 mg/kg diet of BCX-supplemented groups had 64% and 68% decreases, respectively, of *il-6* mRNA level compared with the NNK + Nic group ( $P < 0.05$ ; Fig. 4E). The levels of cyclin D1 and p50 proteins in the NNK + Nic were lower, but the levels of *egr1* mRNA were higher than that of the control group ( $P < 0.05$ ; Fig. 4F–H). There were no differences in the levels of cyclin D1 protein, p50 protein, and *egr1* mRNA between the BCX-supplemented groups and the control group (Fig. 4F–H).

### BCX restores the SIRT1 protein levels

The 10 and 20 mg/kg diet of BCX-supplemented groups had increased levels of the SIRT1 protein, by 91% and 67%, respectively, compared with the NNK + Nic group (Fig. 5A;  $P < 0.01$ ). However, *sirt1* mRNA levels in the lungs were similar across all groups (Fig. 5B).

### BCX supplementation decreases AKT phosphorylation and induces the mRNA levels of both *p53* and *RAR- $\beta$*

The 10 and 20 mg/kg diet of BCX-supplemented groups had decreases of phosphorylated AKT levels, by 65% and 58%, respectively, as compared with the NNK + Nic group (Fig. 5C). There were restored lung levels of tumor suppressor *p53* and *RAR- $\beta$*  mRNA in the BCX-supplemented groups to their normal levels, as observed in the control group (Fig. 5D and E).

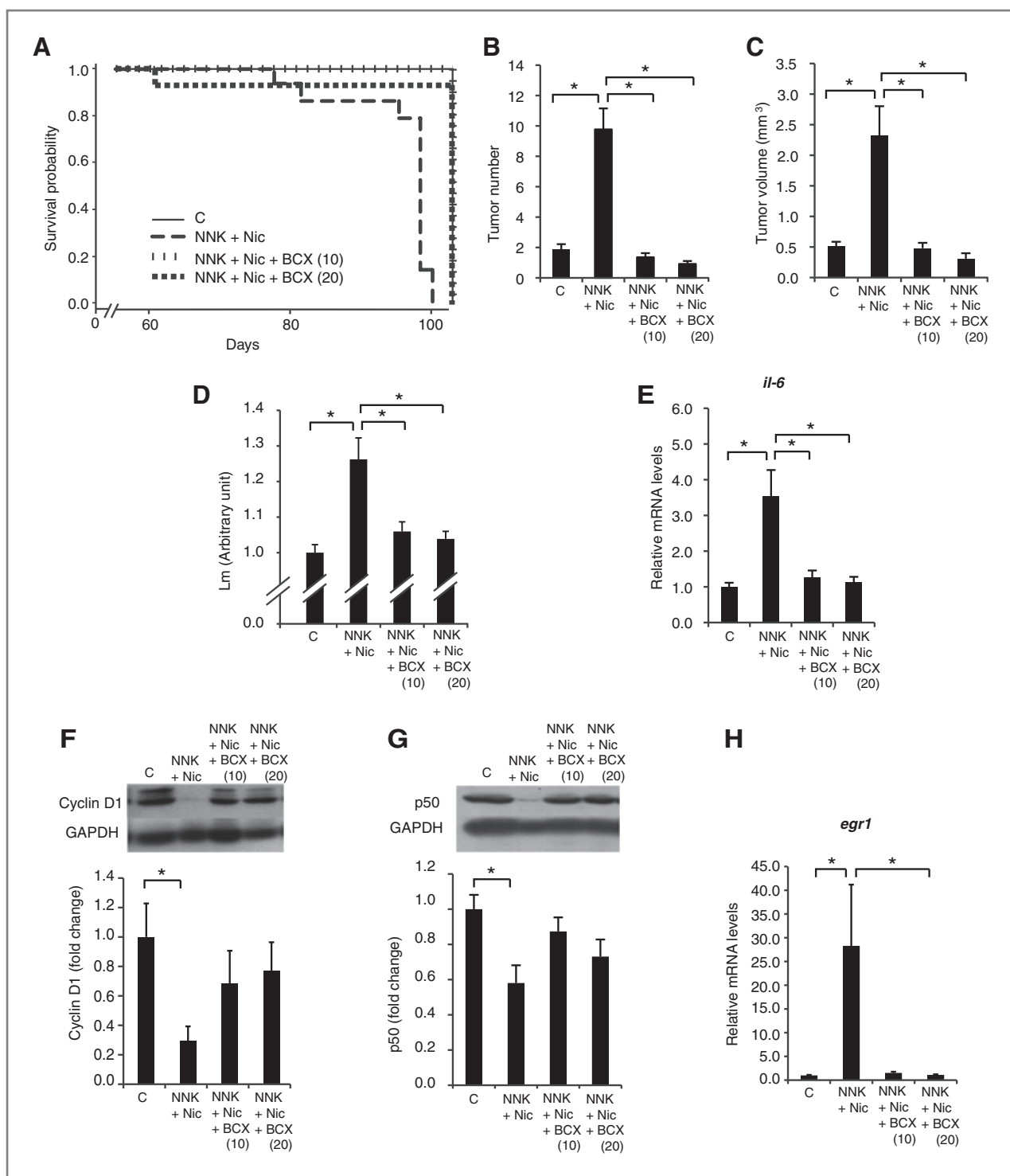
## Discussion

This study shows that nicotine exposure not only increases lung tumor multiplicity and size but also induces emphysema in the A/J lung cancer mouse model. To our knowledge, this is the first model reporting lung tumor development within an emphysematous microenvironment. This study would provide a relevant animal model mimicking lung cancer patient with emphysema. The daily dose of nicotine was equal to the average cigarette consumption in the United States (23) or to the dose/serving in nicotine replacement therapy products such as gums, inhalers, and lozenges (34). This animal model would be useful in studying the molecular pathways leading to emphysema and lung tumorigenesis associated with smoking and smokeless tobacco products. This model would be valuable in examining potential chemopreventive agents against emphysema and lung cancer.

Using this model, we provided a verification of BCX as a chemopreventive agent that prevented both nicotine-promoted lung tumor and emphysema. The BCX doses were relevant to physiologic levels in humans based on several observations. First, although mice have lower absorption of the intact carotenoids compared with humans (35); we detected a serum concentration of BCX of 9.9  $\mu\text{g/L}$  in A/J mice supplemented with 10 mg BCX/kg diet (unpublished data), which is lower than that of the average U.S. population (77  $\mu\text{g/L}$ ; ref. 36). Second, the mean liver BCX concentration in the mice supplemented with 10 mg BCX/kg diet (0.25 nmol/g) was lower than that in human livers (0.66 nmol/g; ref. 37). Finally, the BCX doses used in this study were equivalent to daily human consumption of 0.87 and 1.74 mg BCX (see Materials and Methods). These doses are slightly higher than that in a cohort of Chinese men (38), in which 0.74 mg BCX/2,000 kcal intake was associated with a lower lung cancer risk (38).

The protective role of SIRT1 against emphysema is supported by previous reports, in which SIRT1 maintains genomic integrity and protects against cigarette smoke-induced emphysema in mice (12). This study showed that the decreased SIRT1 levels were associated with nicotine-induced emphysema and lung tumorigenesis. Moreover, the restoration of SIRT1 protein levels to the normal levels was associated with the protective action of BCX against emphysema and lung tumorigenesis. We found that the downregulation of SIRT1 in the nicotine-treated mice and the upregulation of SIRT1 in the BCX-supplemented mice occurred without changes in *sirt1* mRNA levels, suggesting a posttranslational regulation of *sirt1*. The exact mechanism (s) how the SIRT1 protein levels are regulated by nicotine or BCX needs further investigation, which currently undergoes in our laboratory.

The BCX supplementation was associated with the induction of both the SIRT1 protein and *p53* mRNA levels. This observation is in agreement with a previous study in that double heterozygous *sirt1*<sup>+/-</sup>; *p53*<sup>+/-</sup> mice had a higher incidence of tumors (76%) than heterozygous *sirt1*<sup>+/-</sup> and *p53*<sup>+/-</sup> mice (10% and 13%, respectively; ref. 39). Moreover, the increased SIRT1 levels observed in the

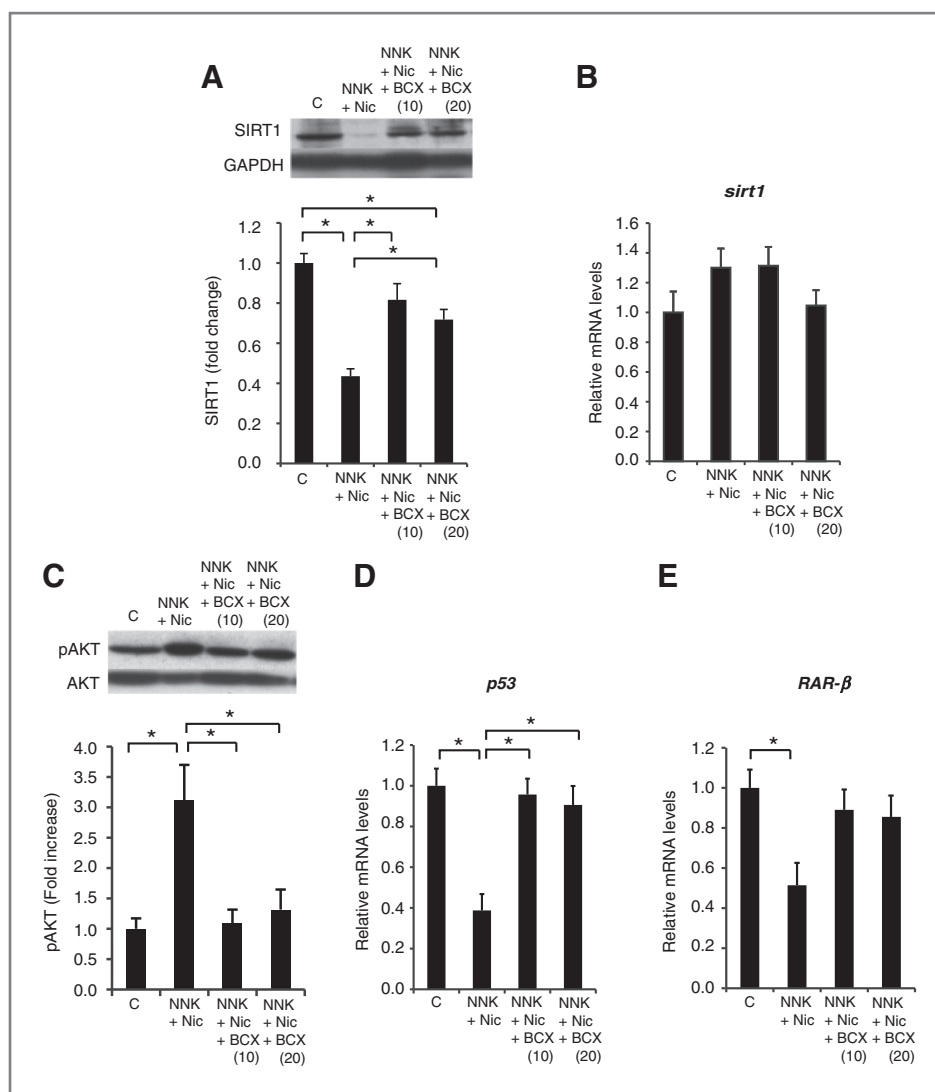


**Figure 4.** BCX supplementation maintains survival probability, decreases lung tumor multiplicity and size, and prevents emphysema. The survival curves for the 3 treatment groups in experiment 2 were analyzed using SAS PROC LIFETEST. A, the log-rank test results for the control, NNK + Nic, NNK + Nic + BCX (10), and NNK + Nic + BCX (20) groups ( $P < 0.01$ ). B, the quantification of the lung tumor multiplicity. C, the number of lung surface tumors and the quantification of the lung tumor volume (mm<sup>3</sup>). D, effects of BCX supplementation on nicotine-induced emphysema and lung inflammation. The degree of emphysema was quantified by calculating the  $L_m$  using 10 fields per animal. E, the lung *il-6* mRNA levels. F, the lung cyclin D1 protein levels. Inset, a representative image of the cyclin D1 and GAPDH proteins. G, the lung p50 protein levels. Inset, a representative image of the p50 and GAPDH proteins. H, the lung *egr1* mRNA levels. The proteins were detected using Western blotting. The intensity of the bands was quantified using a densitometry and normalized to GAPDH levels. The mRNA levels were measured using quantitative real-time PCR (qRT-PCR) and normalized to  $\beta$ -actin mRNA levels. The columns represent the mean  $\pm$  SEM. \*,  $P < 0.05$ . BCX (10),  $\beta$ -cryptoxanthin at 10 mg/kg diet; BCX (20),  $\beta$ -cryptoxanthin at 20 mg/kg diet; C, control; *egr1*, early growth response 1.

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**Figure 5.** BCX restores the SIRT1 protein level and the transcription of tumor suppressor genes and decreases the phosphorylation of AKT. A, the lung SIRT1 protein levels. Inset, a representative image of the SIRT1 and GAPDH proteins. B, the lung *sirt1* mRNA levels. C, the quantification of AKT phosphorylation on Ser473. Inset, a representative image of pAKT and total AKT. The mRNA levels of (D) *p53* and (E) *RAR-β*. The proteins were detected using Western blotting. The intensity of the bands was quantified using densitometry and normalized to GAPDH or total AKT levels. The mRNA levels were measured using quantitative real-time PCR (qRT-PCR) and were normalized to *β-actin* mRNA levels. The columns represent the mean ± SEM. \*, *P* < 0.05. BCX (10), β-cryptoxanthin at 10 mg/kg diet; BCX (20), β-cryptoxanthin at 20 mg/kg diet; C, control.



BCX-supplemented groups were associated with increased *RAR-β* mRNA. This observation can be explained by a previous observation in that SIRT1 coactivates *RAR-β* (40), which itself is capable to regulate its transcription (i.e., *RAR-β* mRNA; ref. 41). Therefore, the induction of SIRT1 in the BCX-supplemented mice may explain the increased *RAR-β* transcription.

In this study, the reduced SIRT1 protein levels were associated with elevated *il-6* mRNA. These findings are consistent with a previous study showing that homozygous *sirt1*<sup>-/-</sup> mice exhibited increased lung levels of both *IL-6* and TNF-α following exposure to fine air particulates (42). Although we could not obtain the data on *IL-6* protein due to the limited mouse lung samples, a direct correlation between mRNA and protein levels of cytokines has been reported (43, 44). The increase of *il-6* mRNA in the NNK + Nic group was also associated with an induction of *egr1* mRNA. EGR1 has been implicated in emphysema as it regulates genes important for extracellular matrix remodel-

ing and those encoding cytokines/chemokines (45). Both *egr1* transcript and EGR1 protein levels were increased in patients with advanced emphysema (45), supporting our observation in which *egr1* mRNA levels were increased in the NNK + Nic group that exhibited emphysema. We also found that the increased *il-6* levels were associated with lower NF-κB subunit p50 protein levels. This observation is inconsistent with the notion that an activation of NF-κB increases the transcription of inflammatory cytokines (13). However, our data are in agreement with a previous study in which *p50*<sup>-/-</sup> knockout mice develop emphysema (46). Earlier reports have described that the p50 protein is inactive because it lacks a transactivation domain and that the formation of p50/p50 homodimer binds to corepressor complexes that are capable in repressing the NF-κB-dependent transcription of inflammatory genes (46).

The decreased cyclin D1 protein level in the NNK + Nic group was in contrast to what we expect in tumorigenesis as the NNK + Nic group had increased lung tumor volume.

Increased cyclin D1 level is found in many human tumors, reflecting an increase in cell proliferation. However, we believe that this reduced cyclin D1 protein could be due to decreased capability of alveolar repair/regeneration in emphysema (47). For example, fibroblasts obtained from emphysematous lungs have a reduced proliferation rate (50%–60% reduction) as compared with fibroblasts from normal human lungs (48). This notion is consistent with our observation in that cyclin D1 levels in the BCX-supplemented groups were restored to the control group.

We observed the highest AKT phosphorylation levels in the NNK + Nic group, which had the lowest survival probability compared with the NNK and control groups. This result is consistent with a previous report in which tumors of patients with non-small cell lung cancer displaying increased AKT phosphorylation levels that were associated with lower survival compared with tumors displaying lower AKT phosphorylation levels (49). Furthermore, the increased SIRT1 levels observed in the BCX-supplemented groups were associated with lower AKT phosphorylation levels. This observation is supported by previous studies reporting an inverse association between SIRT1 and AKT phosphorylation (50); the loss of SIRT1 protein resulted in higher AKT phosphorylation levels in mouse embryonic fibroblasts (50).

Because BCX is a pro-vitamin A carotenoid, this study raises an important question whether its protective effects resulted from BCX as an intact molecule or from its metabolites. Our study suggests that the action of BCX against lung tumor and emphysema is independent of its metabolite vitamin A (retinol and retinoic acid) activity due to the following factors. First, unlike the pro-vitamin A carotenoid  $\beta$ -carotene, for which supplementation at 120 mg/kg diet resulted in no antitumor effects in NNK-treated male A/J mice (22), we show here that BCX supplementation at 10 and 20 mg/kg diet was effective. Second, we observed that in contrast to  $\beta$ -carotene, which has no effect or even detrimental effects on lung tumorigenesis at a high-dose  $\beta$ -carotene in a ferret model (51), BCX supplementation decreased cigarette smoke-induced lung inflammation and precancerous lesions in the same ferret model (18). Third, treatment with the derivative of vitamin A *all-trans* retinoic acid (RA) at 0.5 to 2.5 mg/bw i.p. does not reverse cigarette

smoke-induced emphysema in A/J mice (52) and treatment with *all-trans* RA at 0.5 mg/bw i.p. does not alter cigarette smoke-induced emphysema in guinea pigs (53). Fourth, treatment with 9-*cis* RA (7.5, 15, and 30 mg 9-*cis* RA/kg diet) causes loss of body weight in NNK-treated A/J mice (29), yet, BCX did not. Finally, BCX itself affects the transcription activity of RAR (17, 54). BCX has a greater effect on the transcription activity of RAR than other carotenoids (i.e., astaxanthin, lutein,  $\beta$ -carotene, zeaxanthin, and lycopene; ref. 54). Therefore, this study suggests a new avenue of research about the function of BCX independent of its metabolites.

### Disclosure of Potential Conflicts of Interest

Any opinions, findings, conclusions, and recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the sponsors. No potential conflicts of interest were disclosed.

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