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Randomized phase II clinical trial of sulforaphane in former smokers at high risk for lung cancer

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Abstract

Experimental studies have shown dietary isothiocyanates reduced cellular proliferative marker Ki-67 and increased apoptotic markers Caspase-3 and TUNEL in animals, but human data are lacking. The present study was to assess whether sulforaphane would stop/reverse the progression of bronchial histopathology, reduce Ki-67 index and/or increase Caspase-3 and TUNEL indices in humans. A randomized clinical trial (NCT03232138) was conducted in former smokers. Forty-three subjects were randomly assigned to the placebo or the treatment with a potential daily dose of 95 μmol sulforaphane for 12 months. The endpoints were the changes of histopathology scores, and Ki-67, Caspase-3 and TUNEL indices in post- *vs.* pre-treatment bronchial biopsies. Thirty-

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seven participants (17 in the sulforaphane and 20 in the placebo group) completed the study. Supplementation of sulforaphane did not show significant impact on bronchial histopathology, but significantly reduced Ki-67 index with a 20% decrease in the sulforaphane group and a 65% increase in the placebo ($p = 0.014$). The difference was even greater in high-density (3+) positive Ki-67, with a 44% decrease in the sulforaphane group compared with a 71% increase in the placebo ($p = 0.004$). Higher bioavailability of sulforaphane was correlated with greater reduction of Ki-67 index (P for trend = 0.019). Sulforaphane treatment had no impact on Caspase-3 or TUNEL index in bronchial biopsies. No severe adverse event was observed in the study participants. The findings of oral sulforaphane that significantly reduced Ki-67 index in bronchial tissue support further development as a potential chemopreventive agent against lung cancer development.

INTRODUCTION

Lung cancer is the leading cause of cancer death in both men and women worldwide. It is estimated that lung cancer caused 1.8 million deaths worldwide (1) and 135,000 deaths in the U.S. in 2020 (2). Cigarette smoking accounts for approximately 90% of lung cancer burden in the U.S. (3). Smoking cessation is the most effective means to reduce lung cancer incidence and mortality. Smoking cessation programs and antismoking campaigns in the past half a century have resulted in significant reduction in the prevalence of cigarette smoking in the U.S., from more than 50% of men smoking cigarettes in 1965 (4) to 13% in 2021 (5). There have been more former smokers than current smokers in the U.S. since 2000 (6). However, former smokers remain at high risk of lung cancer even decades after they quit smoking (7). With increasing number of people who quit smoking, more cases of lung cancer are being diagnosed among former smokers who quit smoking for many years, even decades. Given the lack of preventive approaches for lung cancer, there is an urgent need to develop effective, inexpensive, widely available cancer preventive strategies for primary prevention of lung cancer, particularly for those who quit smoking but remain at high risk for lung cancer.

Consumption of cruciferous vegetables has been found to be associated with reduced risk of lung cancer in multiple populations (8, 9). Isothiocyanates derived from cruciferous vegetables have demonstrated anti-cancer properties (10, 11). A biomarker-based prospective cohort study demonstrated that higher urinary total isothiocyanate was significantly associated with lower risk of lung cancer incidence (12). Experimental studies have shown that dietary isothiocyanates, specifically phenethyl isothiocyanate and sulforaphane, significantly inhibited the incidence of carcinogen-induced lung adenocarcinoma in rodents (13, 14).

Multiple studies have clearly demonstrated that the major mechanism by which the inhibitory effect of phenethyl isothiocyanate on tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung carcinogenesis is through the inhibition of the metabolic activation of NNK by cytochrome P450 enzymes (13, 15–18). Both phenethyl isothiocyanate and sulforaphane also can enhance the levels of

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Phase II enzymes such as glutathione S-transferases that increase the detoxication of tobacco and environmental carcinogens and toxicants (19–22).

Besides the alteration of carcinogen bioactivation and detoxication, the chemoprevention of lung cancer by isothiocyanates may be through their impact on cell cycle regulation. Ki-67 is a proliferation marker expressed in all phases of the cell cycle except in the resting phase (23). Because abnormal epithelial proliferation is a hallmark of tumorigenesis, Ki-67 index could be a surrogate endpoint biomarker (SEBM) for risk of lung cancer. In the mouse experiment described above, ingestion of sulforaphane significantly reduced the number of lung proliferative cells (14). In a double-blinded randomized clinical trial, women with abnormal mammograms and scheduled for breast biopsy who consumed sulforaphane precursor glucoraphanin supplements for 2–8 weeks showed significantly reduced Ki-67 index in breast benign tissue (24). In a study of patients with ground glass nodules detected in computed tomography (CT) scans, Ki-67 expression was correlated with pathologic stages from atypical hyperplasia/ adenocarcinoma in situ (lowest expression), minimally invasive adenocarcinoma, and invasive adenocarcinoma (highest expression) (25). No data are available on the effect of dietary isothiocyanates on Ki-67 in human bronchial biopsies.

Another potential mechanism for the chemoprevention by isothiocyanates may be involved the induction of apoptosis, which results in the deletion of genetically damaged cells, such that clonal expansion is aborted. Numerous animal experimental studies have demonstrated that isothiocyanate and its conjugates can induce apoptosis (26–32). There are no data on the effect of dietary isothiocyanates on apoptotic biomarkers in human bronchial tissue.

It has long been established that abnormal bronchial histology (atypia) is associated with cigarette smoking and increased risk of lung cancer (33, 34). Multiple morphological changes in bronchial epithelia from hyperplasia and metaplasia to dysplasia and carcinoma in situ occur before the manifestation of invasive carcinoma. In a follow-up study of high-risk individuals who underwent bronchoscopy, persistence of bronchial dysplasia was associated with significantly increased risk of developing non-small cell lung cancer (35). These data suggest that histopathological change in bronchial epithelia may be an informative biomarker for the progression or regression of lung lesions that precede the development of lung cancer, which could be utilized for evaluating chemopreventive efficacy.

We evaluated the potential chemopreventive efficacy of sulforaphane against lung carcinogenesis through a randomized, double-blinded, placebo-controlled phase II clinical trial, a proof-of-principal study, with 12 months of treatment for former smokers who were at high risk for lung cancer. We hypothesized that ingestion of sulforaphane improves histopathological features, decreases cell proliferation and increases apoptosis in bronchial tissue. Our ultimate goal is to validate the efficacy of this readily available, widely accessible, inexpensive, natural compound as a primary chemopreventive agent against the development of lung cancer in humans.

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MATERIALS AND METHODS

Study Design

We employed a double-blind, placebo-controlled, randomized clinical trial study design. The study was registered with ClinicalTrials.gov (NCT03232138).

Study Population and Eligibility Criteria

The eligibility criteria were adults aged 55–75 years residing in southwestern Pennsylvania who had a history of smoking with 30 or more pack-years and had quit smoking for at least one year but no more than 10 years, presence of airflow obstruction on spirometry (GOLD 1–4 & FEV1/FVC<0.7 & FEV1< 80% predicted), or chronic obstructive pulmonary disease (COPD) or any emphysema on a previous CT scan, and absence of lung invasive carcinoma or carcinoma in situ in baseline bronchial biopsies. In addition, subjects eligible for the study had no hypercania ($PaCO_2$ < 50 mmHg) and were generally healthy with liver enzymes and blood counts within the following ranges–white blood cell counts ≥ 3,000/mL; blood urea nitrogen (BUN), serum creatinine, and total bilirubin all ≤ 1.5 upper limits of normal (ULN); and both aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ≤ 2.5 ULN. All eligible participants signed written informed consent before any physical/clinical examinations and collection of any data or specimens began. To protect participants' rights, privacy, and welfare, the University of Pittsburgh Internal Review Board (IRB) conducted a thorough review of this study to ensure that the research complied with federal, state, and institutional regulations, including, but not limited to, the Belmont Report, Declaration of Helsinki and the US Common Rule (IRB # STUDY19040278).

Consented patients underwent a baseline bronchoscopy, which included collection of bronchial washing (i.e., lavage), brushing, and biopsies from six predetermined sites (left and right upper lobe orifices and superior segment orifices, carina between left upper lobe division and lingular orifices and right middle lobe orifice) as well as from any abnormal sites suspicious for cancer. A repeated bronchoscopy was performed to collect the same sets of biospecimens at the end of the 12-month treatment. All bronchial biopsies were formalin fixed, paraffin embedded (FFPE), and some of the FFPE slides underwent hematoxylin & eosin (H&E) staining for histopathologic evaluation. At baseline and trimonthly clinical visits, we also repeatedly collected nasal brushing and blood samples for research use as well as for monitoring potential systemic toxicity. RNA was extracted from both bronchial and nasal brushing samples immediately after collection. Blood and lavage samples were processed and made into multiple aliquots. All biospecimens were stored at −80°C for further analysis.

Subject Recruitment and Randomization

Study subjects were recruited from Western Pennsylvania through local advertisement and clinical referrals. Most of the subjects who participated in this clinical trial were also participants of the Pittsburgh Lung Screening Study, a longitudinal cohort of current and former smokers for low-dose CT screening for early detection of lung cancer (36). Figure 1 shows the flow of subject enrollment. The representativeness of the study subjects was presented in Supplementary Table S1. Overall, 105 subjects passed the telephone screening

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for eligibility, and 67 signed consent forms and completed baseline clinical tests for eligibility, and 43 of them were eligible and randomly assigned with an equal chance either to the sulforaphane (n = 21) or placebo arm (n = 22). Four dropouts occurred in the sulforaphane arm due to ineligibility found after randomization (one subject), non-compliance with the study protocol (one subject), or possible adverse events (two subjects) whereas there were two dropouts in the placebo arm (one with a family member's health issue and the other with non-compliance with the study protocol). The originally planned sample size was 80 eligible subjects to be randomly assigned to the two treatment arms. The present study was under recruited due to the emergence of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-COV-2) pandemic beginning in March 2020.

Intervention

Following randomization, each subject was provided with study tablets at months 0, 3, 6, and 9, and instructed to ingest orally four Avmacol® or placebo tablets in the morning with breakfast and four tablets in the evening with dinner over ensuing 3 months. A single Avmacol® tablet contained 15 mg glucoraphanin (the precursor of sulforaphane) along with myrosinase while a placebo tablet contained neither glucoraphanin nor myrosinase (Nutramax Laboratories Consumer Care, Inc., Lancaster, SC). A daily intake of 8 Avmacol® tablets provided 120 mg (274 μmol) glucoraphanin delivering a potential daily dose of 95 μmol sulforaphane assuming a 35% conversion rate according to previously reported conversion rates of 20% to 35% in human studies by others (37, 38). The total intervention period was 12 months. To track the daily intake of study tablets, study participants were asked to keep a diary recording the number of tablets taken, which were reviewed and recorded by research staff at each of the trimonthly clinical visits

Compliance Measurements

The patients' compliance with consumption of cruciferous vegetables and intake of study tablets was monitored through the following activities at each trimonthly visit: (1) self-reported intake of frequency and amount of each of the cruciferous vegetables (i.e., broccoli, bok choy, Brussel sprouts, cabbage, cauliflower, Chinese cabbage or Napa cabbage, kai lan, kai choy, kale, mustard green, radish, turnips, wasabi and watercress); (2) participant diaries recording intake of study tablets; and (3) measurements of urinary sulforaphane and sulforaphane metabolites.

Measurement of sulforaphane and sulforaphane metabolites in urine was performed at the Plants for Human Health Institute, North Carolina State University (GV), using a modification of a previously reported isotope dilution mass spectrometric assay as described by Egner and colleagues (39). Positive ion MS/MS was carried out using a binary gradient on a Shimadzu LCMS-IT-TOF mass spectrometer with a SIL-30AC controller and a CTO-30A column oven maintained at 37°C. Sulforaphane-*d*8 and sulforaphane *d*8-acetylcysteine were used as internal standards. Metabolites monitored were sulforaphane, sulforaphane N-acetyl-L-cysteine, sulforaphane glutathione, sulforaphane-L-cysteine, sulforaphane glucosinolate (glucoraphanin), sulforaphane-glucuronide, sulforaphane-sulfate, and sulforaphane-cysteine-glycine.

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Outcome Measurements

Histopathology Evaluation: Histopathology score was assigned to each of the six tissue slides from 6 predetermined sites according to the 2021 World Health Organization criteria for precursor squamous lesions and non-small cell carcinoma: 1= normal; 2= reserve cell hyperplasia; 3 = squamous metaplasia; 4 = mild dysplasia; 5 = moderate dysplasia; 6 = severe dysplasia; 7 = carcinoma in situ; and 8 = invasive non-small cell carcinoma (40, 41). Subjects with score 7 or 8 at any baseline biopsy were ineligible for the study. All biopsies were graded by the study pathologist (SD) in a blinded fashion as to treatment group.

Endobronchial histopathological scores were first summarized over six biopsies per bronchoscopy for each of three separate endpoints at pre- and post-treatment, respectively. The first endpoint was dysplasia index, i.e., the number of biopsies with dysplasia divided by the total number of biopsies examined. A positive change of 10% or more was considered a progression while a negative change of 10% or more a regressive change. Scores in-between were considered being stable. The second endpoint was the mean histopathological scores over six tissue slides at pre- and post-treatment, respectively. A positive change in the score by 1 or more points was defined as progressive change, a negative change in the score by 1 or more points as a regressive change, and changes in scores in-between were defined as stable condition. The third endpoint was called the “worst score”, which identified the highest score of histopathological grade among six biopsy sites at pre- and post-treatment, respectively. A positive change in the highest score by 1 or more points was defined as progressive condition, a negative change by 1 or more points a regressive condition, and changes in scores in-between a stable condition.

Immunohistochemistry for proliferation and apoptosis: Immunohistochemistry (IHC) was performed for one FFPE section of six biopsy sites with validated antibodies in the CLIA-accredited Clinical IHC Laboratory in Department of Pathology, University of Pittsburgh Medical Center (UPMC). The IHC assays for Ki-67 and cleaved caspase-3 were automated on the Leica Bond III using prediluted Ki-67 antibodies (Dako Catalog #IR626, Carpinteria, CA) and caspase-3 antibodies (Biocare Medical Catalog #PP-229-AA, Pacheco, CA), respectively, following the manufacturer’s instructions. The IHC for TUNEL was performed manually using the Apoptosis Detection Kit (Millipore Catalog #S7100, RRID:AB_2661855, Burlington, MA) following manufacturer’s instruction.

Automated Image Analysis: The IHC-stained slides were scanned on the Aperio AT2 scanner (Leica Biosystems) at 40X magnification. The digital images were uploaded into eSlideManager (Leica Biosystems, version 12.4.3.8020) and annotated using Aperio Imagecope (Leica Biosystems, version 12.4.3.7009). Each image was manually annotated using the pen tool to define the areas for analysis. The areas of non-tissue or poor tissue were annotated with the negative pen tool and excluded from analysis. We analyzed images using the default (i.e. unmodified) Leica/Aperio Nuclear V9 algorithm for Ki-67 and Leica/Aperio Positive Pixel Count V9 algorithm for TUNEL to determine the number of total nuclei with positive staining, as well as number of positive nuclei with low (1+), moderate (2+) and high (3+) intensity of brown staining. For the cytoplasmic assessment of cleaved Caspase-3, we used the Leica/Aperio Cytoplasmic V2 algorithm to determine the percentage

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of cells with positive Caspase-3 staining over total cells in the area of the tissue fragment analyzed. Further, the intensity of brown staining in the cytoplasm were classified into low (1+), moderate (2+) and high (3+)-intensity positives using the Leica/Aperio standard settings. The algorithm also provided a H-score for caspase-3, which was the sum of weighted intensity scores = % low (1+) positive + 2 × % moderate (2+) positive + 3 × % high (3+) positive.

Assessment of Adverse Events

The potential adverse events associated with intake of Avmacol® tablets were monitored by physical examinations and blood tests, and self-reported at each trimonthly clinical visits. The physical examinations included vital signs, weight, carbon monoxide and oxygen level, and lung function by spirometry. Blood tests included complete blood counts, and liver and kidney function tests. A questionnaire was administered to each participant to document any adverse event associated with the intake of Avmacol® according to the terms and grades in the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0 (https://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm).

Statistical Analysis

Before conducting formal statistical analysis for the effect of sulforaphane intake on cellular biomarkers, we calculated the average values of total cleaved Caspase-3, Ki-67 and TUNEL of 6 sites at pre- and post-treatment, respectively, for each study participant. We also calculated means of these IHC markers by their intensity of staining.

The two-group *t*-test (for continuous variables) or Fisher exact or chi-square statistics (for discrete or nominal variables) were used to assess the differences between the two treatment groups. Analysis of covariance (ANCOVA) method was used to assess a difference-in-differences in means of endobronchial histopathological scores and IHC markers between two treatment groups with adjustment for following covariates at baseline: age, sex, number of cigarettes per day, number of years of smoking, and number of years since last smoked, and the baseline value of the respective outcome measurements. In subgroup analysis, subjects were grouped according to their median levels of summed urinary total sulforaphane metabolites throughout the active intervention period.

Statistical analyses were carried out using SAS software version 9.3 (SAS Institute, Cary, NC). All *P* values reported are two-sided, and those of <0.05 were considered to be statistically significant.

Data Availability

Summary data generated in the study are available within the article and its supplementary materials. Deidentified individual-level data is available through the corresponding author.

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RESULTS

Basic Observations

Baseline demographics and lifestyle factors of study subjects are presented in Table 1. The sulforaphane group included more men, younger subjects, and persons with fewer years of quitting smoking. There were no statistically significant group differences in level of smoking, alcohol consumption, presence of airflow obstruction, CT-detected emphysema and COPD as well as histories of cardiovascular disease, hypertension and type 2 diabetes (Supplementary Table S2).

Compliance

Participants in the sulforaphane and placebo groups reported mean (standard deviation [SD]) daily consumption of total cruciferous vegetables of 0.23 (0.28) and 0.30 (0.27) cups, respectively ($p = 0.459$) at baseline. The consumption of cruciferous vegetables remained low and constant through the 12-month study period with daily mean (SD) of 0.20 (0.17) cups in the sulforaphane group and 0.29 (0.21) cups in the placebo group ($p = 0.153$) (Supplementary Table S3).

Compliance measured by urinary total sulforaphane metabolites was shown in Table 2. The mean (SD) urinary total sulforaphane metabolites at months 3 and 6 were 261.95 (178.72) and 250.10 (217.74) μmol/g creatinine, which was 400–590 times that of placebo group. Sulforaphane-acetylcysteine was the most abundant metabolite detected (77.6%), followed by unconjugated sulforaphane (14.3%) and sulforaphane-cysteine (8.0%); whereas the precursor glucosinolate (glucoraphanin) was not detectable in any urine samples tested. Although the levels of urinary total sulforaphane metabolites decreased over time during the intervention period due to lower compliance according to self-reported intake of study tablets provided, the sulforaphane group still had approximately 75- to 190-fold higher urinary content of total sulforaphane metabolites than the placebo group in the late months (9 and 12) of the intervention period.

Outcome Analysis

In the placebo group, 14 subjects (70%) had squamous metaplasia as the highest (i.e. worst) histopathology score among the 6 bronchial biopsies, 4 (20%) had reserve cell hyperplasia, and 2 (10%) had normal epithelia at baseline. The corresponding numbers in the sulforaphane group were 10 (58.8%), 6 (35.3%), and one (5.9%). None of the biopsies from participants at baseline in either the sulforaphane or placebo group showed any dysplasia or more severe lesions. After treatment with sulforaphane for 12 months, there was one (5.9%) subject with mild dysplasia as the worst histopathology score, 9 (52.9%) with squamous metaplasia, 3 (17.6%) with reserve cell hyperplasia, and 4 (23.5%) with normal epithelia. The corresponding numbers in the placebo group after 12 months were 10 (50%) with squamous metaplasia, 8 (40%) with reserve cell hyperplasia and 2 (10%) with normal epithelia, but none with dysplasia (Supplementary Table S4).

The worst endobronchial histopathology scores in post-treatment decreased by ≥ 1 point in 5 (29.4%) participants, remained the same in 8 (47.1%), and increased by ≥ 1 point in 4

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(23.5%) in the sulforaphane group (Table 3). The corresponding figures in the placebo group were 7 (35.0%), 9 (45.o%) and 4 (20.0%), respectively ($P = 0.928$).

The mean (SD) endobronchial histopathology scores over six biopsy sites at baseline were 1.67 (0.38) in the sulforaphane group and 1.62 (0.42) in the placebo group ($P = 0.696$). The mean scores decreased by 0.22 in the sulforaphane group and 0.12 in the placebo group ($P = 0.452$) (Table 3).

The mean Ki-67, TUNEL and cleaved Caspase-3 indices in baseline bronchial biopsy samples were comparable between the sulforaphane and placebo groups (all P's > 0.19) (Table 4). After treatment, the overall average numbers of Ki-67 positive nuclei per mm^2 over 6 biopsy samples decreased by 5.27 (95% CI −18.13–7.59) or 20% in the sulforaphane group whereas it increased by 18.03 (95% 7.18–28.88) or 65% in the placebo group ($P = 0.014$). When Ki-67 positive nuclei were counted by intensity, the difference between two treatment groups was greatest for high-intensity with a 44% decrease in the sulforaphane and a 71% increase in the placebo group ($P = 0.004$), followed by moderate-intensity (a 17% decrease *vs.* a 64% increase, P = 0.028), and weakest for low-intensity (a 4% decrease *vs.* a 61% increase; $P = 0.056$). Overall, there were no statistically significant differences in pre- versus post-treatment TUNEL and cleaved Caspase-3 between two treatment groups (Table 4).

We also conducted subgroup analyses for subjects in the sulforaphane group by median (180 μmol/g creatinine) of the summed urinary sulforaphane metabolites. There was a dose-response relation for higher levels of sulforaphane metabolites with a greater reduction in Ki-67 index overall (P trend = 0.019) (Figure 2). This inverse trend was strongest for high-intensity (3+) positive Ki-67 nuclei (P trend = 0.008), followed by moderate intensity (P trend = 0.022) and low intensity (P trend = 0.069). Urinary levels of sulforaphane metabolites were not associated with the change of TUNEL or cleaved Caspase-3 (Supplementary Table S5).

Adverse events

Total number of adverse events by attribution and severity reported by all randomized study participants during the observed study period are shown in Supplementary Table S6. Overall there were 107 adverse events in the sulforaphane group and 100 adverse events in the placebo group. Among these adverse events, 98.1% were mild or moderate whereas only one in the sulforaphane and three in placebo group were severe, but all of them were judged to be unrelated to the study agent. By attribution, 74.9% of total adverse events were unrelated to the study intervention, 22.2% possibly related, and only 2.9% probably related. All of these probably related adverse events were mild or moderate in severity. There were no statistically significant differences in number of subjects with adverse events between the two treatment groups (both P's > 0.13). Overall, 18 (85.1%) of the 21 subjects in the sulforaphane group and 20 (90.9%) of the 22 subjects in the placebo group reported gastrointestinal adverse events including flatulence, followed by constipation, diarrhea and dry mouth (P = 0.664). There were 16 (71.4%) subjects in the sulforaphane group and 15 (68.2%) subjects in the placebo group who reported neurological adverse events such as dizziness, headache and mood change (P = 1.00) (Supplementary Table S7). The extensive

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blood chemistries for liver and kidney function tests, and blood cell counts were performed in trimonthly blood samples and did not reveal any abnormal values.

DISCUSSION

This randomized, placebo-controlled, phase II clinical trial demonstrates that oral supplementation of 8 Avmacol® tablets delivering a potential daily dose of approximately 95 μmol sulforaphane (assuming a 35% conversion rate) for 12 months significantly decreased Ki-67 index in bronchial biopsies compared with placebo among former smokers. The effect of sulforaphane on Ki-67 was greatest for high-intensity positive staining and in subjects with higher compliance with sulforaphane intake. These findings provide supportive evidence for using Ki-67 as a potential SEBM for lung cancer risk in the evaluation of as a potential chemopreventive agent. As mentioned earlier, a randomized clinical trial also demonstrated that dietary supplementation of sulforaphane precursor significantly reduced Ki-67 index in breast benign tissue (24). Ki-67 was also used as a SEBM in other two prior clinical trials that demonstrated a significant reduction of bronchial Ki-67 index in both former and current smokers after oral treatment with Celecoxib, a Cox-2 inhibitor, for 3–6 months (42, 43). Unfortunately, the increase in cardiovascular adverse events associated with Celecoxib prevented further evaluation in phase III clinical trials. More studies are warranted to evaluate Ki-67 index as a SEBM in cancer chemopreventive trials.

Cigarette smoking, a primary cause of lung cancer, can directly cause oxidative stress (44). Previously a number of large, randomized placebo-controlled phase III trials were conducted to assess the efficacies of antioxidants for prevention of lung cancer development. Two earliest Phase III trials – the Beta-Carotene and Retinol Efficacy Trial (CARET) and the Alpha-Tocopherol and the Beta-Carotene Cancer Prevention Study (ATBC Study) – showed no beneficial effect but an adverse effect, in which heavy smokers or those exposed to asbestos assigned to the treatment with beta-carotene in combination with alpha-tocopherol or retinol had a statistically significant 16% and 28% higher incidence of lung cancer than those assigned to the placebo, respectively (45, 46). Additional large, randomized phase III trials with supplementation of antioxidants such as beta-carotene, selenium, and vitamins C and E showed null effect on lung cancer risk, although these studies were not designed for lung cancer as primary endpoints (47–50). In retrospect, the failure of these large phase III trials might be due to a lack of understanding about anti-carcinogenic mechanisms of antioxidants, especially for heavy smokers who already were at high risk for lung cancer.

Following the failure of large phase III trials on antioxidant agents, more phase II trials with smaller sample sizes and shorter durations of intervention have been conducted to test promising chemopreventive agents, particularly those with anti-inflammatory properties since cigarette smoking can also trigger inflammation (44). Based on the gradual and stepwise changes of histopathology in bronchial epithelia towards lung cancer, these phase II trials focused on premalignant histopathological change. Two phase II trials evaluated the efficacy of inhalational corticosteroid agents (i.e., budesonide and fluticasone) with anti-inflammatory properties, and neither one showed any significant impact on regression or progression of bronchial metaplasia and/or dysplasia after treatment for 6 months (51, 52). A third phase II trial tested iloprost, a prostacyclin analogue, and found that oral

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treatment for 6 months significantly reduced average score of bronchial histopathology, the worst histopathology, and dysplasia index in former smokers, but not in current smokers (41). A more recent phase Ib trial also demonstrated that inhaled iloprost for 60 days significantly upregulated genes that were in immune response and downregulated genes in cell proliferation pathways (53). These data support that iloprost could be further developed as a potential lung chemopreventive agent, although its oral formula is currently unavailable.

In the present study, we used Avmacol® tablets that contained both glucoraphanin and myrosinase. After ingestion, the myrosinase catalyzes the conversion of glucoraphanin to sulforaphane. This formula has been found to provide consistent in vivo delivery of 20% to 35% of the glucoraphanin content as sulforaphane (37, 38). After absorption, sulforaphane is conjugated with glutathione and quickly accumulates in various types of cells. Sulforaphane is a potent inducer of the cytoprotective enzyme NAD(P)H quinone dehydrogenase 1 and other carcinogen detoxication enzymes, which are regulated principally through the Keap1-Nrf2-Antioxidant-Response Element signaling pathway (54). Indeed, earlier clinical trials in Qidong, China, with glucoraphanin/sulforaphane-rich broccoli sprout extracts demonstrated enhanced detoxication and urinary excretion of metabolites of benzene (a human carcinogen) and acrolein (20, 55). Recently, a study with a 2-week administration of Avmacol® daily showed enhanced detoxication of benzene, acrolein and crotonaldehyde in current smokers (56).

The present study did not show any significant change on regression or progression of bronchial histopathology after 12-month treatment with sulforaphane. One of the possible explanations for null results might be due to lower baseline histopathology score. Intuitively a high grade of histopathology, e.g., dysplasia, at baseline would be required to potentially show any regressive effect after an intervention treatment. In our study, baseline bronchial dysplasia was not one of the eligibility criteria due to the invasive bronchoscopy procedure and every low prevalence of dysplasia in former smokers. Instead old age, history of heavy smoking, presence of airflow obstruction, and presence of COPD and emphysema were used, but failed to enrich high-risk former smokers with bronchial dysplasia.

It is noted that there was one subject whose squamous metaplasia at baseline progressed to mild dysplasia at the end of the 12-month treatment with sulforaphane. The subject was a 65 year-old man with 48 pack-years of smoking who quit smoking for one year before he was enrolled into the study. The IHC measurements from pre- to post-treatment were 22.9 to 33.6 (a 47% increase) for Ki-67, 4.1 to 3.2 (a 22.0% decrease) for TUNEL, and 4.1 to 1.5 (a 63.9% decrease) for cleaved Caspase-3. These data suggested that a histopathological progression was coincided with increased cell proliferation and decreased apoptotic biomarkers.

Our study did not show any significant impact of sulforaphane on TUNEL or Caspase-3 in bronchial biopsies. The discrepancy in these IHC biomarkers between experiments in animals and humans may be due to the differences in species, dosages and duration of treatment with sulforaphane by body size and life expectancy.

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The present study showed that daily ingestion of 8 Avmacol® tablets containing 120 mg (274 μmol) glucoraphanin for 12 months were safe and free of severe adverse events. As expected, most common adverse events were in gastrointestinal and neurological systems. All reported adverse events were mild. The extensive laboratory tests trimonthly did not reveal any abnormal values in liver and kidney function tests or blood cell counts. This (lack of) toxicity profile is consistent with the notion that cruciferous vegetables are part of routine diet and safe. The absence of severe adverse events in our study is also consistent with previous human studies with similar doses (20, 39, 57).

Similar to other phase II clinical trials, our study has limitations. Due to the pandemic of SARS-CoV-2 during the course of our trial, the enrollment of study participants was extremely challenging, especially for the requirement of bronchoscopy at baseline and the end of the intervention period, which resulted in lower accruals than that planned. The small sample size produced unequal sampling in our study with differences in age, gender, pack-years of smoking and years of quitting smoking. Higher level of smoking history might have contributed to the notable increase in Ki-67 index in the placebo group. Although these variables were adjusted in the statistical analysis, the residual confounding on endpoints could not be completely ruled out. The SARS-CoV-2 pandemic also caused shutdown of our research clinic and disrupted regular clinical visits of participants for toxicity and compliance monitoring, especially the later part of the study period, with lower intake of study tablets reported and decreased levels of urinary sulforaphane metabolites tested. A stronger treatment effect of sulforaphane on Ki-67 index in participants with higher levels of urinary sulforaphane metabolites suggests that it is critically important for the compliance with intake of study agents.

In summary, this 12-month randomized, placebo-controlled phase II clinical trial demonstrates that oral supplementation of Avmacol® tablets delivering a potential daily dose of approximately 95 μmol sulforaphane is safe and effective on reducing the number of cells with positive Ki-67 in bronchial biopsies in former smokers. Further clinical trials with larger sample size and longer duration of intervention and follow-up are warranted to evaluate its efficacy against the development of lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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PREVENTION RELEVANCE

High intake of cruciferous vegetables and their sulforaphane are associated with lower incidence of lung cancer in humans and animal models. This clinical trial has demonstrated that oral supplementation of sulforaphane for 12 months significantly reduced Ki-67 index, a potential surrogate endpoint of biomarker for lung cancer risk.

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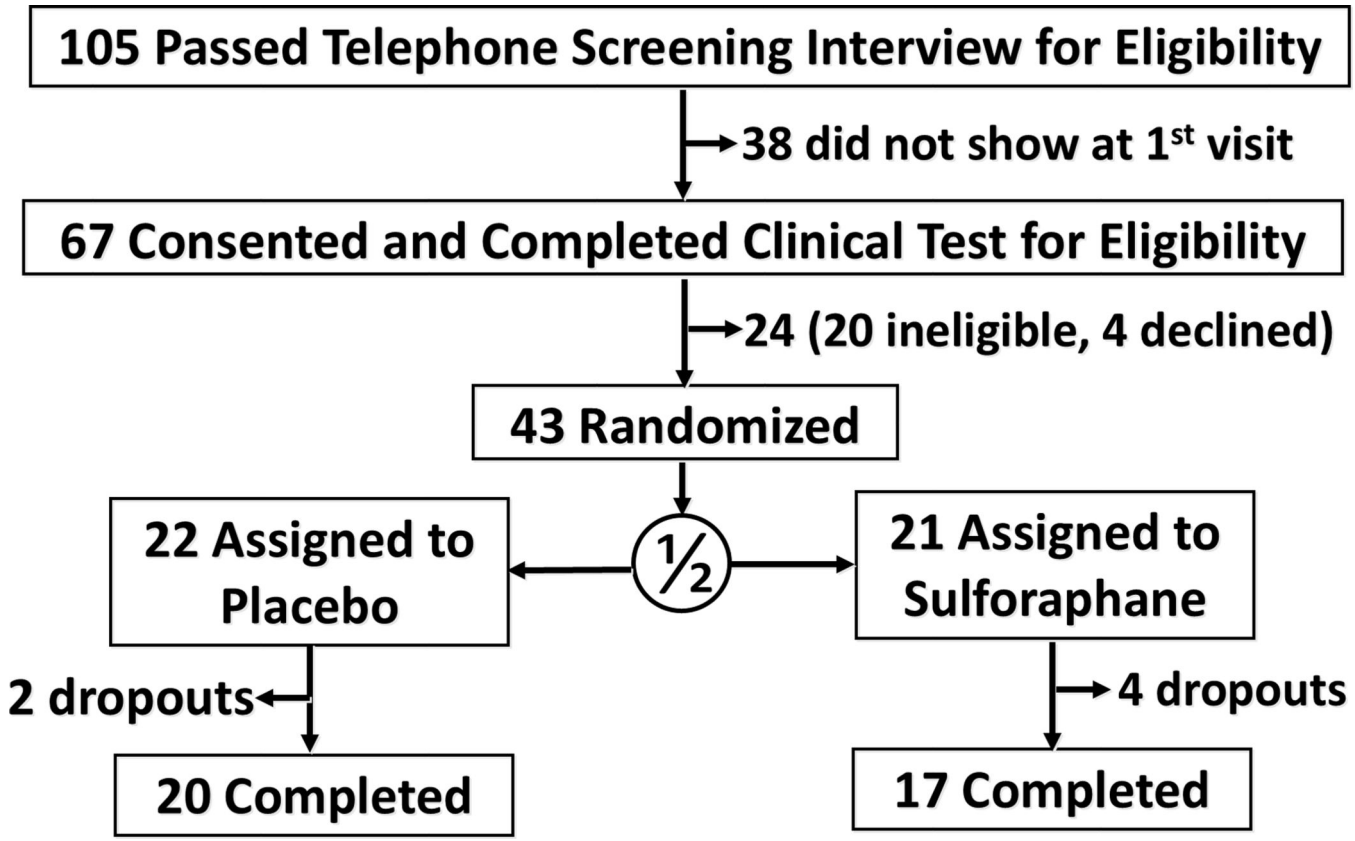


Figure 1. Profile of subject flow in the Pittsburgh Clinical Trial of Sulforaphane on Risk Markers of Lung Cancer

Of the 43 eligible subjects, 21 were randomly assigned to the sulforaphane arm and 22 to the placebo arm. Four dropouts occurred in the sulforaphane arm due to ineligibility found after randomization (one subject), non-compliance with the study protocol (one subject), or possible adverse events (two subjects) whereas two dropouts occurred in the placebo arm - one with a family member's health issue and the other with non-compliance with the study protocol.

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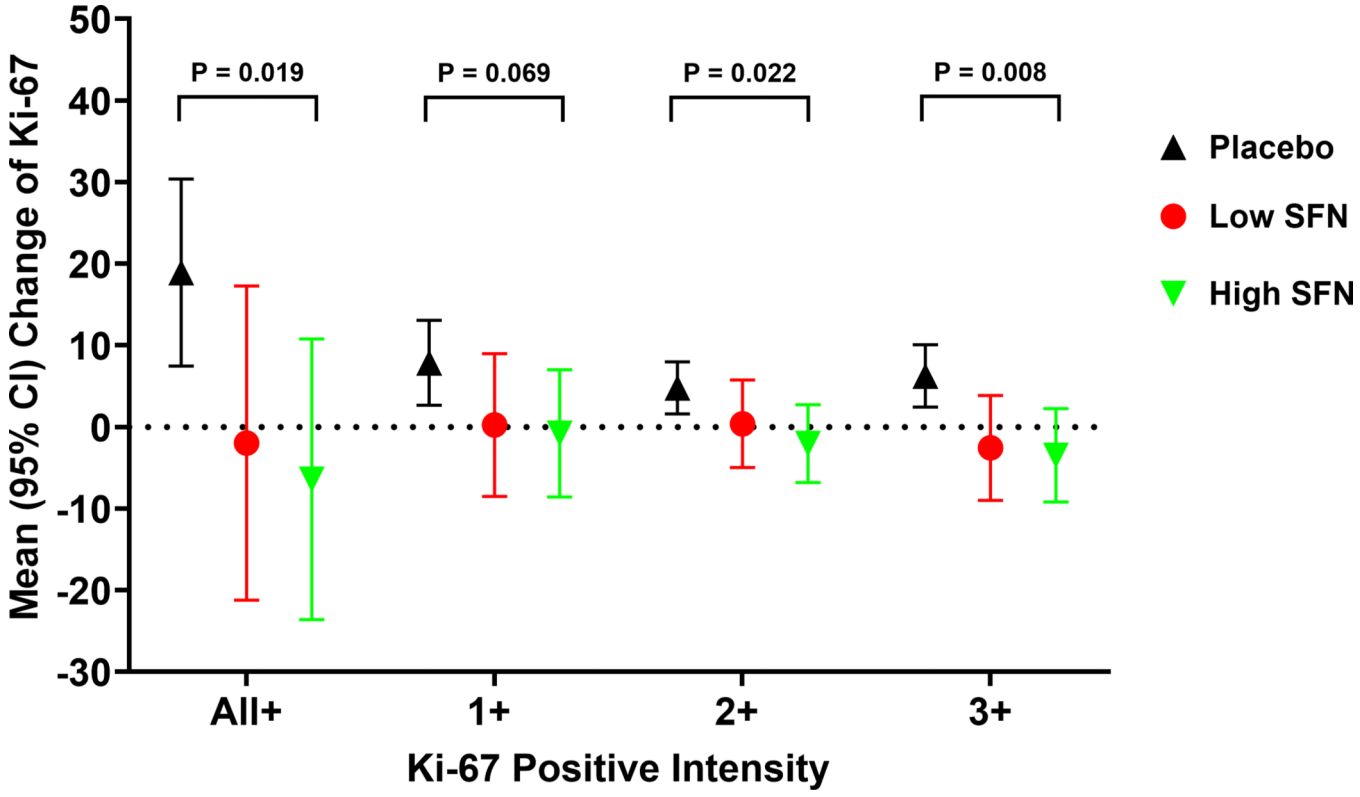


Figure 2. Mean changes of Ki-67 indices overall and by intensity level after oral supplementation of sulforaphane for 12 months, the Pittsburgh Clinical Trial of Sulforaphane on Risk Markers of Lung Cancer

Higher levels of sulforaphane metabolites was associated with a greater reduction in Ki-67 index overall (P trend = 0.019), and strongest for high-intensity (3+) positive Ki-67 nuclei (*P* trend = 0.008), followed by moderate intensity (*P* trend = 0.022) and low intensity (*P* trend = 0.069).

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Table 1.

Distributions of selected demographic and lifestyle factors at baseline by treatment group, The Pittsburgh Clinical Trial of Sulforaphane on Risk Markers of Lung Cancer

Baseline factors	Sulforaphane (n = 17)	Placebo (n = 20)	P value*
Age (years), mean (SD)†	64.1 (5.3)	68.0 (3.2)	0.010
Men, N (%)	13 (76.5)	9 (45.0)	0.052
White, N (%)	17 (100)	19 (95.0)	1.0
Body mass index (kg/m^2), mean (SD)	30.6 (6.1)	28.6 (4.2)	0.249
Level of education, N (%)			0.715
High school or less	3 (17.6)	5 (25.0)	
College	12 (70.6)	11 (55.0)	
Graduate school	2 (11.8)	4 (20.0)	
Marital status, N (%)			0.575
Married	10 (58.8)	8 (40.0)	
Never married	1 (5.9)	3 (15.0)	
Divorced/Widowed	6 (35.3)	9 (45.0)	
History of cigarette smoking			
No. of cigarettes/day, mean (SD)	23.8 (10.2)	27.5 (8.4)	0.244
Years of smoking, mean (SD)	39.5 (8.2)	42.2 (4.5)	0.209
Year of quit smoking, mean (SD)	4.3 (2.9)	6.8 (3.2)	0.019
Pack-years of smoking, mean (SD)	48.3 (29.5)	58.3 (29.5)	0.227
Alcohol drinking, N (%)			0.200
Non-drinkers	2 (11.8)	7 (35.0)	
< 7 drinks/week	13 (76.4)	10 (50.0)	
7+ drinks/week	2 (11.8)	3 (15.0)	
Pulmonary function test			
FEV1 % predicted, mean (SD)	72.8 (13.8)	75.0 (14.7)	0.640
FEV1/FVC ratio, mean (SD)	0.62 (0.10)	0.64 (0.08)	0.591
Airflow obstruction measures			0.741
GOLD I	4 (23.5)	7 (35.0)	
GOLD II	12 (70.6)	12 (60.0)	
GOLD III	1 (5.9)	1 (5.0)	
GOLD IV	0	0	
Presence pf emphysema on CT scan			0.212
No	5 (29.4)	2 (10.0)	
Yes	12 (70.6)	18 (90.0)	
History of COPD			0.969
No	5 (29.4)	6 (30.0)	
Yes	12 (70.6)	14 (70.0)	
Family history of lung cancer			0.745

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Baseline factors	Sulforaphane (n = 17)	Placebo (n = 20)	P value*
No	10 (58.8)	13 (65.0)	
Yes	7 (41.2)	7 (35.0)	

SD, standard deviation.

* Derived from *t*-test or *Chis-square* or *Fisher* exact test.

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Table 2.

Urinary total sulforaphane metabolites by treatment group during the study period The Pittsburgh Clinical Trial of Sulforaphane on Risk Markers of Lung Cancer

Time points after initial of treatment	Mean (SD) of urinary total sulforaphane metabolites* (μmol/mg creatinine)	
	Sulforaphane (N = 16†)	Placebo (N = 20)
Month 3	248.23 (207.12)	0.42 (0.55)
Month 6	263.22 (249.50)	0.65 (1.53)
Month 9	120.71 (108.38)	1.69 (2.94)
Month 12	139.29 (175.24)	0.45 (1.09)

SD, standard deviation.

*Total sulforaphane metabolites measured included sulforaphane, sulforaphane N-acetyl-L-cysteine, sulforaphane glutathione, sulforaphane-L-cysteine, sulforaphane glucosinolate, sulforaphane-glucoronide, sulforaphane-sulfate, and sulforaphane-cysteine-glycine.

†One subject was excluded from this analysis due to missing data on measurements of urinary total sulforaphane metabolites.

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Table 3.

Endobronchial histopathological scores in pre- and post-treatment bronchial biopsy samples by treatment group, The Pittsburgh Clinical Trial of Sulforaphane on Risk Markers of Lung Cancer

Endobronchial histopathological scores	Sulforaphane (n = 17)	Placebo (n = 20)	P value (treatment effect)
Change of the highest score, N (%)*			0.928†
Lower	5 (29.4)	7 (35.0)	
No change	8 (47.1)	9 (45.0)	
Higher	4 (23.5)	4 (20.0)	
Average score of 6 biopsies			
Pre-treatment, mean (SD)	1.67 (0.38)	1.62 (0.41)	0.696‡
Post-treatment, mean (SD)	1.55 (0.44)	1.48 (0.37)	0.590‡
Mean change from pre- to post-treatment (95% CI)	0.22 (0.03, 0.41)	0.12 (−0.04, 0.28)	0.452‡

SD, standard deviation.

*The highest endobronchial histological score among 6 biopsies per participant

†Derived from *Chi-square* test.

‡Derived from Analysis of Covariant that also included baseline average endobronchial histopathological scores, age, sex, number of cigarettes per day, number of years of smoking, and number of years since quit smoking.

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Table 4.

Means and changes of cellular proliferative and apoptotic biomarkers in 6 pre- and 6 post-treatment endobronchial biopsies by treatment group, The Pittsburgh Clinical Trial of Sulforaphane on Risk Markers of Lung Cancer

Cellular biomarkers in bronchial biopsies	Baseline means (SD)			Mean (95% CI) changes from pre- to post-treatment		
	SFN (n = 17)	Placebo (n = 20)	P value*	SFN (n = 17)	Placebo (n = 20)	P value†
Ki-67 positive nuclei (counts/mm^2)						
All positive nuclei	26.07 (14.73)	27.75 (15.40)	0.738	-5.27 (−18.13, 7.59)	18.03 (7.18, 28.88)	0.014
Weak-intensity positive nuclei	11.71 (5.52)	12.21 (6.05)	0.780	-0.50 (−6.44, 5.26)	7.48 (2.55, 12.41)	0.056
Moderate-intensity positive nuclei	6.70 (4.93)	7.22 (4.33)	0.733	-1.16 (−4.76, 2.44)	4.61 (1.57, 7.64)	0.028
Strong-intensity positive nuclei	7.67 (4.81)	8.32 (7.17)	0.751	-3.41 (−7.67, 0.85)	5.93 (2.24, 9.52)	0.004
TUNEL positive nuclei (counts/μm^2)						
All positive nuclei	5.01 (1.50)	4.57 (1.49)	0.377	-0.21 (−0.96, 0.53)	0.35 (−0.27, 0.96)	0.291
Weak-intensity positive nuclei	2.73 (0.75)	2.51 (0.85)	0.413	-0.05 (−0.43, 0.34)	0.12 (−0.20, 0.44)	0.552
Moderate-intensity positive nuclei	1.78 (0.55)	1.61 (0.51)	0.338	-0.10 (−0.38, 0.18)	0.16 (−0.08, 0.39)	0.205
Strong-intensity positive nuclei	0.50 (0.29)	0.45 (0.24)	0.547	-0.05 (−0.18, 0.09)	0.05 (−0.06, 0.17)	0.295
Caspase-3 positive cytoplasm (% of total cells examined)						
All positive cells	4.46 (3.89)	2.96 (4.58)	0.295	-1.28 (−2.64, 0.08)	-1.00 (−2.15, 0.15)	0.778
Weak-intensity positive cells	4.18 (3.65)	2.69 (3.90)	0.242	-1.19 (−2.44, 0.06)	-0.83 (−1.88, 0.23)	0.685
Moderate-intensity positive cells	0.25 (0.31)	0.25 (0.65)	0.985	-0.07 (−0.21, 0.08)	-0.14 (−0.26, −0.02)	0.469
Strong-intensity positive cells	0.04 (0.11)	0.02 (0.05)	0.547	-0.01 (−0.02, −0.001)	-0.03 (−0.04, −0.02)	0.052
H-Score‡	4.79 (4.20)	3.26 (5.31)	0.343	-1.38 (−2.88, 0.12)	-1.20 (−2.46, 0.07)	0.866

CI, confidence interval; SD, standard deviation; SFN, sulforaphane.

*Derived from *t*-test.

†Derived from Analysis of Covariance that also included age, sex, number of cigarettes per day, number of years of smoking, number of years of quit smoking, and baseline values of the same cellular biomarker.

‡H-score = weak-intensity positives (%) + 2 × moderate-intensity positives (%) + 3 × strong-intensity positives (%).