



## Original Article

# Prolonged sulforaphane treatment does not enhance tumorigenesis in oncogenic K-ras and xenograft mouse models of lung cancer

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## Abstract

**Background:** Sulforaphane (SFN), an activator of nuclear factor erythroid-2 related factor 2 (Nrf2), is a promising chemopreventive agent which is undergoing clinical trial for several diseases. Studies have indicated that there is gain of Nrf2 function in lung cancer and other solid tumors because of mutations in the inhibitor Kelch-like ECH-associated protein 1 (Keap1). More recently, several oncogenes have been shown to activate Nrf2 signaling as the main prosurvival pathway mediating ROS detoxification, senescence evasion, and neoplastic transformation. Thus, it is important to determine if there is any risk of enhanced lung tumorigenesis associated with prolonged administration of SFN using mouse models of cancer. **Materials and Methods:** We evaluated the effect of prolonged SFN treatment on oncogenic K-ras (K-ras<sup>LSL-G12D</sup>)-driven lung tumorigenesis. One week post mutant-K-ras expression, mice were treated with SFN (0.5 mg, 5 d/wk) for 3 months by means of a nebulizer. Fourteen weeks after mutant K-ras expression (K-ras<sup>LSL-G12D</sup>), mice were sacrificed, and lung sections were screened for neoplastic foci. Expression of Nrf2-dependent genes was measured using real time RT-PCR. We also determined the effect of prolonged SFN treatment on the growth of preclinical xenograft models using human A549 (with mutant K-ras and Keap1 allele) and H1975 [with mutant epidermal growth factor receptor (EGFR) allele] nonsmall cell lung cancer cells. **Results:** Systemic SFN administration did not promote the growth of K-ras<sup>LSL-G12D</sup>-induced lung tumors and had no significant effect on the growth of A549 and H1975 established tumor xenografts in nude mice. Interestingly, localized delivery of SFN significantly attenuated the growth of A549 tumors in nude mice, suggesting an Nrf2-independent antitumorigenic activity of SFN. **Conclusions:** Our results demonstrate that prolonged SFN treatment does not promote lung tumorigenesis in various mouse models of lung cancer.

**Keywords:** EGFR, Keap1, K-ras, lung cancer, Nrf2, sulforaphane

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## INTRODUCTION

Epidemiological studies during the past decade have shown a positive correlation between the general consumption of cruciferous vegetables and decreased incidence of some cancers, including lung,<sup>[1]</sup> stomach, colon, breast,<sup>[2-3]</sup> bladder,<sup>[4-5]</sup> prostate,<sup>[6-7]</sup> and non-Hodgkin

lymphoma.<sup>[8]</sup> Lung cancer risk is reduced especially by dietary intake of isothiocyanates or cruciferous vegetables in persons with genotypes of Glutathione S-transferase Mu 1-null and Glutathione S-transferase theta 1-null, highlighting an important interplay between genetic susceptibility factors and chemopreventive agents.<sup>[1, 9-10]</sup>

The cancer chemopreventive effect of cruciferous vegetables is due to glucosinolates. Sulforaphane (SFN) is formed by enzymatic transformation of a glucosinolate, glucoraphanin, present in cruciferous vegetables, during chewing or through conversion through the gut microflora.<sup>[11]</sup> SFN acts as a chemopreventive agent by inducing phase-II enzymes by activating the nuclear factor erythroid-2 related factor 2 (Nrf2) pathway.<sup>[12-13]</sup> Evidence is mounting that SFN acts through other chemopreventive mechanisms such as blocking cancer initiation via inhibition of cytochrome P-450-dependent monooxygenases that convert procarcinogens into active carcinogens and modulating signaling pathways that regulate cell growth and apoptosis to suppress cancer progression.<sup>[14-15]</sup> Several groups have reported that SFN is effective in preventing chemical carcinogen-induced breast,<sup>[11, 16]</sup> stomach,<sup>[17]</sup> pancreas, and colon cancer,<sup>[18]</sup> as well as decreased polyp formation in *Apc<sup>min</sup>* mice.<sup>[19]</sup> In the lungs, SFN and its *N*-acetylcysteine conjugate inhibit malignant progression of adenomas induced by tobacco carcinogens.<sup>[20]</sup> In addition to its anticarcinogenic effect, SFN treatment inhibits the growth of established prostate,<sup>[21-23]</sup> breast,<sup>[24]</sup> pancreas,<sup>[25-27]</sup> and lung cancer<sup>[28]</sup> xenografts in mice. Preclinical study results have also shown that SFN-mediated activation of Nrf2 improves bacterial clearance and corticosteroid sensitivity in mice with chronic cigarette smoke exposure.<sup>[29-30]</sup> Several clinical trials are underway for evaluating SFN for treatment of various diseases ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)).

Somatic loss of function mutations in Kelch-like ECH-associated protein 1 (Keap1) and oncogenic gain of function mutations in Nrf2 have been reported in lung,<sup>[31]</sup> prostate,<sup>[32]</sup> skin, esophagus,<sup>[33]</sup> gallbladder,<sup>[34]</sup> and ovarian cancer.<sup>[35]</sup> More recently, oncogenic activation of K-ras, Myc, and B-raf have been shown to activate Nrf2 signaling, and this cross talk has been shown to be important for oncogene-induced senescence evasion and tumorigenesis.<sup>[36-37]</sup> For future development of SFN as a chemopreventive or therapeutic agent, we have evaluated the risk of long-term SFN administration on lung tumorigenesis by using oncogenic K-ras<sup>LSL-G12D</sup> mouse model and human lung cancer xenograft mouse models.

## MATERIALS AND METHODS

### Sulforaphane

D, L-Sulforaphane was purchased from Toronto Research

Chemicals, Ontario (Cat#S699115) and stored at -20°C. The SFN was freshly diluted in phosphate-buffered saline (PBS) before treatment.

### Cell culture and reagents

A549 and H1975 cells were purchased from American Type Culture Collection (Manassas, Virginia, United States) and cultured under recommended conditions. Cells were cultured in Dulbecco modified eagle medium (Invitrogen, Carlsbad, California, United States) containing 10% fetal bovine serum and 1% penicillin streptomycin. Ad-CMV-Cre ( $1 \times 10^{10}$  PFU/mL) was obtained from Vector Biolabs.

### Mice

Kras<sup>LSL (G12D)</sup> (strain number 01XJ6)<sup>[38]</sup> mice were obtained from a mouse repository (NCI, Frederick) and were bred in our facility. Athymic *Ncr-nu/nu* (strain number 01B74) nude mice (6-8 weeks) were obtained from NCI. Mice were fed the 2018SX diet (Harlan Teklad) and had access to water ad libitum; they were housed under controlled conditions (23±2°C; 14-h light/ 10-h dark cycles). All experimental protocols conducted on the mice were performed in accordance with National Institutes of Health guidelines and were approved by the Johns Hopkins University Animal Care and Use Committee.

### Effect of SFN on mutant K-ras-mediated lung tumorigenesis

Eight-week-old Kras<sup>LSL-G12D</sup> mice<sup>[38]</sup> were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight). Five million adenovirus cre-recombinase particles diluted in 100 µl of minimal essential medium were administered by oropharyngeal aspiration (50 µl, twice at an interval of 5 min) according to the standard protocol.<sup>[39]</sup> Five days after adenovirus Cre treatment, mice were randomly placed into one of the two groups. SFN (0.5 mg) suspended in 50 µl of PBS was administered 5 d/wk for 14 weeks by using a nebulizer (Aeroneb Lab) in the SFN group ( $n = 9$ ). PBS was administered to the control group ( $n = 9$ ). One mouse from each group was sacrificed at 8 weeks to assess tumor progression. The remaining mice were euthanized at 14 weeks. The lungs were inflated with 0.6% agarose and fixed in 10% formalin. Five longitudinal equidistant serial sections were stained with hematoxylin and eosin stain. The lesions were assessed subjectively for the percentage of lesion area to total lung section area. The lesions were categorized as mild hyperplasia, severe hyperplasia, adenoma, or adenocarcinoma.

### Effect of SFN on the growth of lung cancer xenografts in nude mice

Either A549 cells ( $5 \times 10^6$ ) or H1975 cells ( $1 \times 10^6$ ) were injected subcutaneously into the right flank of the

anesthetized athymic nude mice. When the volume of the tumor reached  $50 \pm 20 \text{ mm}^3$ , the mice were separated randomly into different treatment groups, and SFN treatment was started. The mice were weighed and the tumor dimensions were measured with calipers once a week until 1 week before harvesting of the tumor. The tumor volumes were calculated by using the following formula: length (mm)  $\times$  width (mm)  $\times$  width (mm)  $\times$  0.52.

The four experiments depicted in Table 1 were conducted. SFN injections were administered either intraperitoneally or subcutaneously 5 d/wk. The subcutaneous SFN injections were administered 0.5–1 cm adjacent to the left side of the tumor. The control mice in all the experiments received the same volume of PBS as did their respective cohorts. The mice were euthanized by means of cervical dislocation, and the tumors were removed without skin and weighed. The H1975 tumors were weighed without intratumoral fluid.

#### Real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from tissues and cells by using the RNeasy kit (Qiagen) and was quantified by means of ultraviolet absorption spectrophotometry. The reverse transcription reaction was performed by using a high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, California, United States) and the MultiScribe First Strand Synthesis system (Applied Biosystems, Foster City, California, United States) in a final volume of 20  $\mu\text{l}$  containing 1  $\mu\text{g}$  of total RNA, 100 ng of random hexamers, 1 $\times$  reverse transcription buffer, 1 mM deoxyribonucleotide triphosphate, MultiScribe reverse transcriptase, and nuclease-free water. A quantitative real-time polymerase chain reaction analysis of *NAD(P)H dehydrogenase, quinone 1 (NQO1)*, *heme Oxygenase 1 (HO-1)*, and *glutamate-cysteine ligase, catalytic subunit (GCLC)* was performed by using assay-on-demand primers and probe sets from Applied Biosystems. Assays were performed by using the ABI 7000 TaqMan system.  $\beta$ -actin was used for normalization.

#### Statistical analysis

Tumor volume, tumor weight, and body weight were measured and presented as mean plus or minus standard error and compared by means of a *t*-test or ANOVA. A *P*-value of less than 0.05 was considered statistically significant.

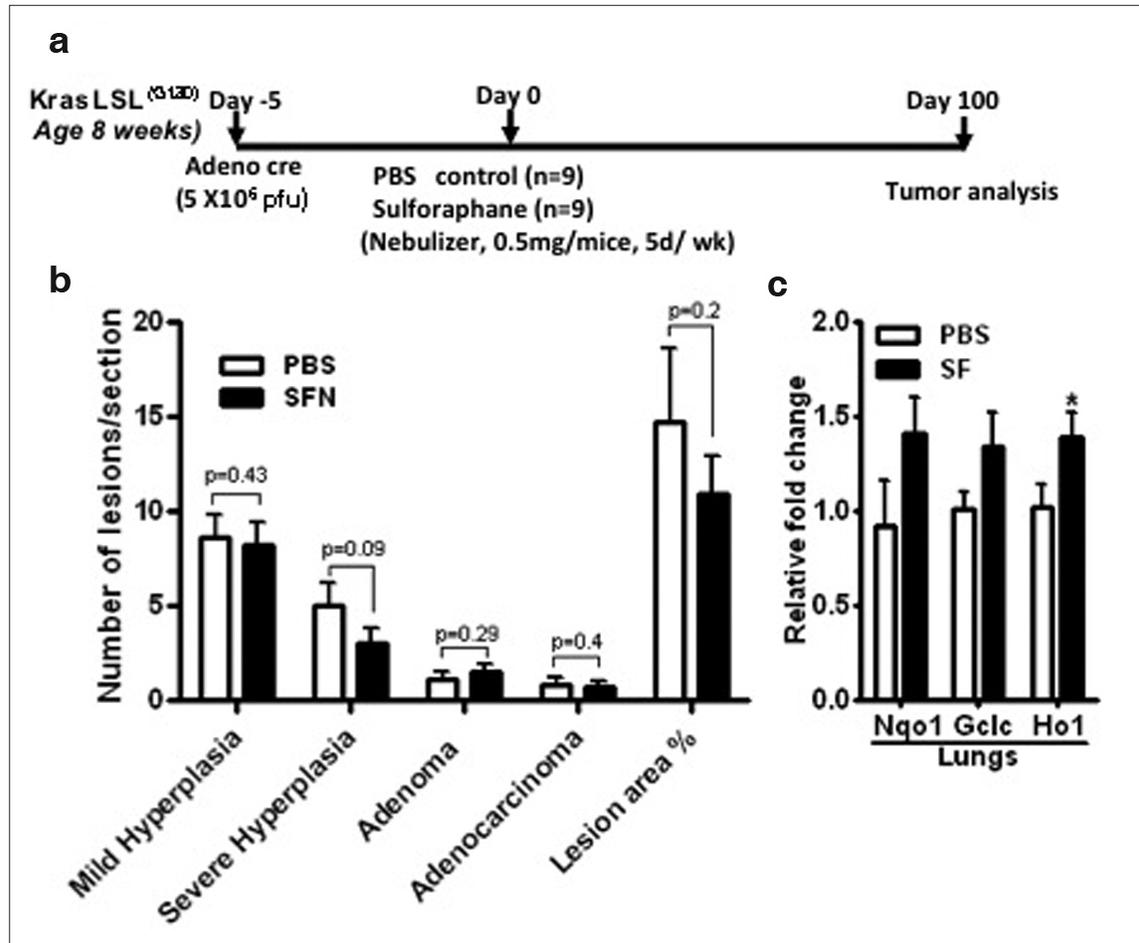
## RESULTS

#### SFN does not enhance mutant K-ras-driven lung tumorigenesis

We determined whether chronic SFN administration affects oncogenic K-ras (K-ras<sup>LSL-G12D</sup>)-driven lung tumorigenesis. The K-ras<sup>LSL-G12D</sup> mutant mouse develops progressively less differentiated lung tumors after the administration of adenoviral Cre-recombinase.<sup>[38]</sup> In this study, we evaluated the effect of SFN on K-ras<sup>LSL-G12D</sup> mediated lung tumorigenesis post oncogenic K-ras activation. One week after administration of a high titer adenoviral Cre to induce mutant K-ras expression, 1 cohort of mice was treated with SFN (0.5 mg, 5 d/wk) for 3 months by means of a nebulizer [Figure 1a]. We have reported that SFN (0.5 mg/mice) administered using nebulizer significantly induces Nrf2-dependent gene expression in lungs.<sup>[30]</sup> The control group was treated with PBS. There was no adverse effect of SFN treatment on animal health or body weight. Fourteen weeks after adenovirus-Cre-recombinase administration and mutant K-ras activation, mice were sacrificed, and lung sections were screened for neoplastic foci. Histological analyses of the lung sections from the K-ras<sup>LSL-G12D</sup> mutant mice showed the presence of atypical adenomatous hyperplasia and adenomas with infiltration of inflammatory cells. Although tumor number, histological subtypes, and grades were not significantly different between the two groups, we noticed a trend toward reduced tumor burden in the SFN-treated cohort of mice [Figure 1b]. Extended exposure to SFN transcriptionally induced the expression of the Nrf2 target genes NQO1, GCLC, and HO-1 in the lungs [Figure 1c]. Thus, our data demonstrate that prolonged exposure to SFN via aerosol inhalation does not promote or increase tumorigenesis in the mutant K-ras<sup>LSL-G12D</sup>-driven mouse model of lung tumorigenesis.

**Table 1: Design for xenograft experiments**

Experiment	Lung cancer xenograft	SFN injections (5 d/wk)	SFN dose	Duration of treatment (weeks)
1	A549 ( $5 \times 10^6$ cells)	Intraperitoneal	3 $\mu\text{mol}$ or 6 $\mu\text{mol}$ in 250 $\mu\text{l}$ PBS (120 or 240 $\mu\text{mol}/\text{kg}$ of body weight)	7
2	A549 ( $5 \times 10^6$ cells)	Subcutaneous	1000 nmol in 100 $\mu\text{l}$ PBS (40 $\mu\text{mol}/\text{kg}$ of body weight)	7
3	A549 ( $5 \times 10^6$ cells)	Subcutaneous	500 nmol in 100 $\mu\text{l}$ PBS (20 $\mu\text{mol}/\text{kg}$ of body weight)	6
4	H1975 ( $1 \times 10^6$ cells)	Intraperitoneal	9 $\mu\text{mol}$ in 250 $\mu\text{l}$ PBS (360 $\mu\text{mol}/\text{kg}$ of body weight)	4



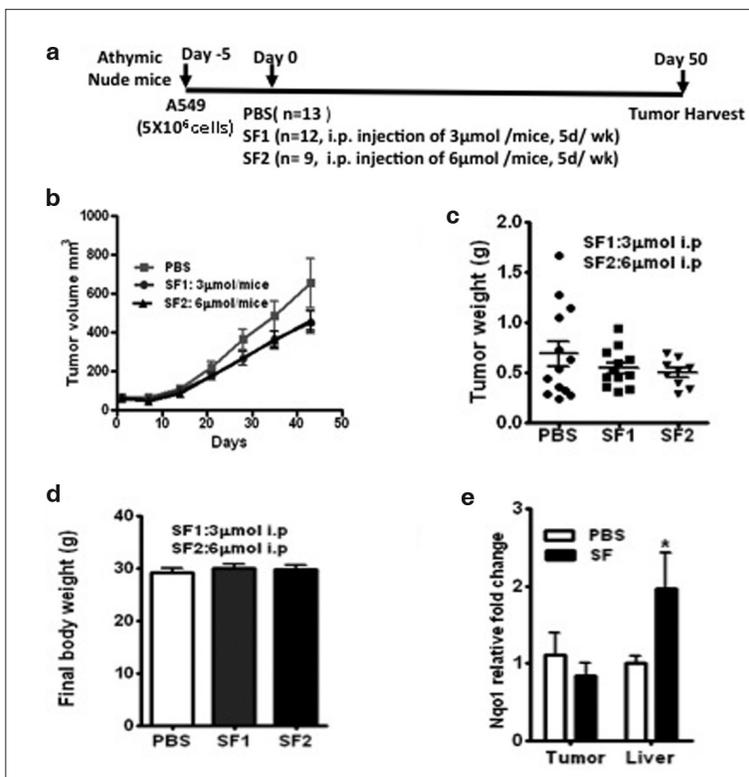
**Figure 1: SFN does not promote oncogenic K-ras-driven lung tumorigenesis. (a) Schematic of the experimental design. (b) Bar graph showing relative number of neoplastic lesions (hyperplasia, adenoma, adenocarcinoma) and percentage of the area of the lung covered by the tumor cells. (c) Real-time RT-PCR data showing induction of Nrf2-dependent gene expression in the lungs of the SFN-treated cohort of mice. P-values are calculated using t test**

### Prolonged systemic SFN treatment does not promote growth of A549 or H1975 lung cancer xenografts

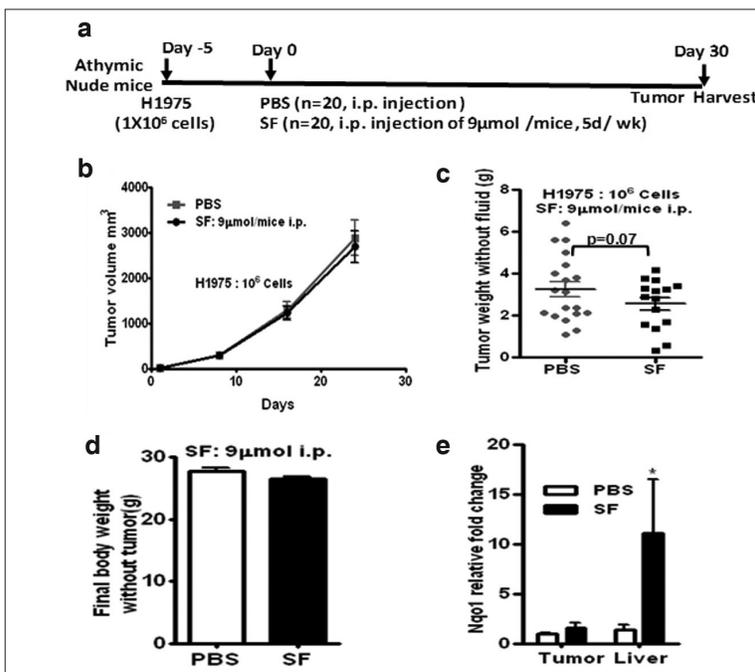
To evaluate the effect of prolonged SFN treatment on the growth of established lung cancer xenografts, we selected two cell lines, A549 and H1975. A549 cells with a mutant K-ras and Keap1 allele, and H1975 with a mutant epidermal growth factor receptor (EGFR) allele, are the two commonly used lung cancer xenograft models. A549 and H1975 cells were injected into the flanks of nude mice, and change in tumor volume was recorded. Once the tumor volume reached  $50 \pm 20 \text{ mm}^3$ , mice were randomly allocated into two groups, and SFN treatment was initiated [Figures 2a and 3a]. Mice were treated with vehicle (PBS) or SFN by means of intraperitoneal injection (5 d/wk), and tumor volume was measured weekly. SFN dose was selected based on previously published results where SFN administration at a dose 250–400  $\mu\text{mol/kg}$  body weight, has been reported to be well tolerated.<sup>[25, 40]</sup> We did not notice a significant reduction in the tumor growth rate/weight of A549 xenografts treated with 3  $\mu\text{mol}$  dose of

SFN, and thus, we increased the SFN dose and repeated the experiment with 3 and 6  $\mu\text{mol}$ . Again, SFN treatment at a dose of 3 or 6  $\mu\text{mol}$  did not inhibit the growth of A549 tumors significantly, although we noticed a trend toward lower tumor volume and weight in the SFN-treated group [Figures 2b and c and 3b and c]. For H1975 cells expressing mutant EGFR allele, we increased the SFN dose further (9  $\mu\text{mol}$ ) since these tumors proliferate rapidly *in vivo*. Similar to our results with A549 tumors, SFN at a dose 9  $\mu\text{mol}$  did not attenuate the growth of H1975 xenografts significantly, although we noticed a trend toward lower tumor volume and weight in the SFN-treated group [Figures 3b and 3c]. To ascertain whether systemic SFN administration causes weight loss, we recorded body weights of the control and treated mice. Average body weights of the control and SFN-treated mice did not differ significantly throughout the treatment protocol [Figures 2d and 3d].

To demonstrate that systemic delivery of SFN induced Nrf2-



**Figure 2: Systemic delivery of SFN does not promote growth of Keap1 and K-ras mutant, A549, lung cancer xenografts. (a) Schematic of the experiment. (b) Graph showing relative growth of A549 cells in nude mice. Tumor volume is not significantly different between the three cohorts of mice (ANOVA). (c) Tumor weights are not significantly different between the three groups. (d) Total body weights are not significantly different between the three groups. (e) Real-time RT-PCR showing expression of the Nrf2-dependent gene, NQO1, in the liver and tumor tissues.\**P* < 0.05 (t test).**



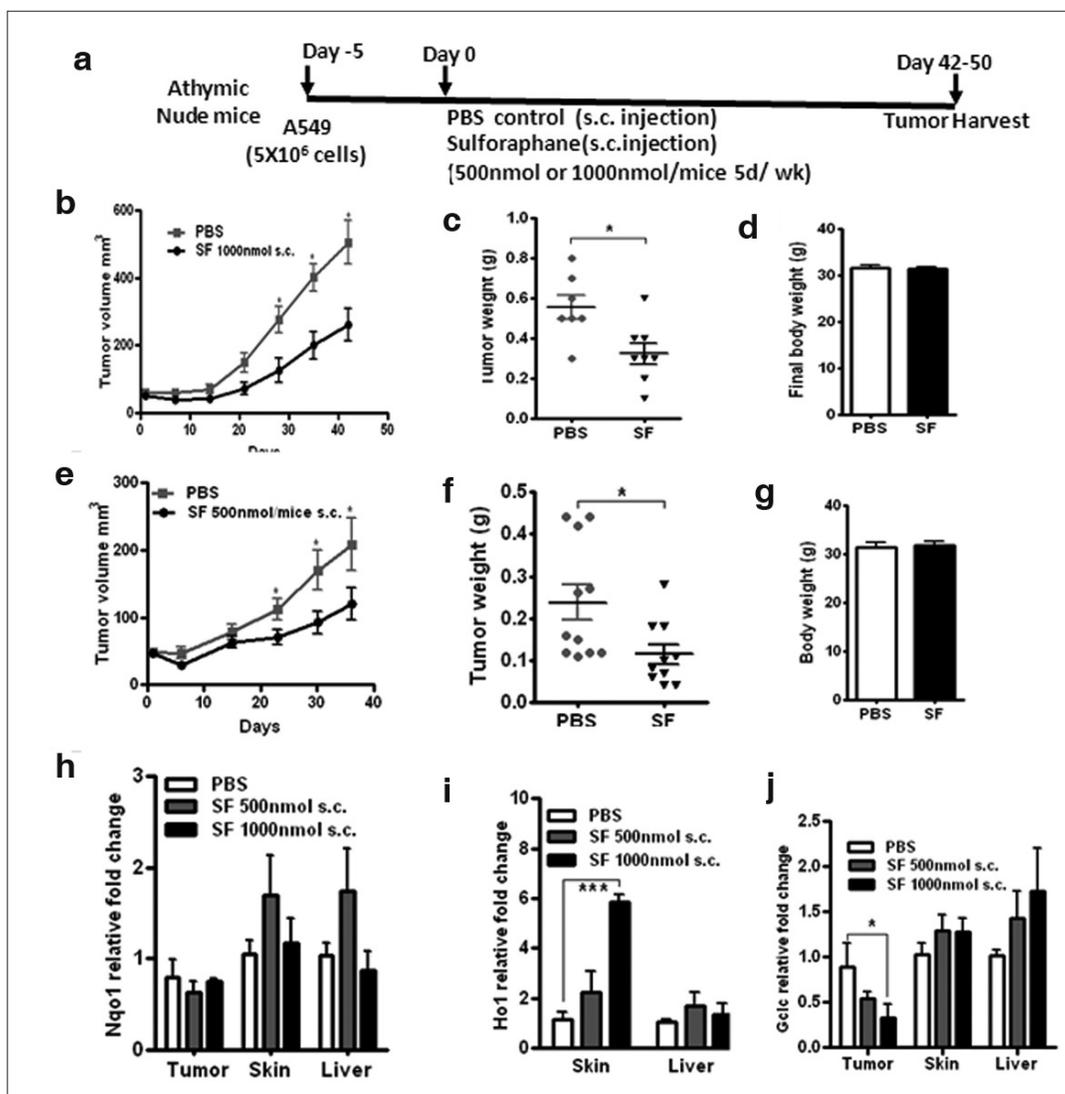
**Figure 3: Systemic delivery of SFN does not promote growth of EGFR mutant (H1975) lung cancer xenografts. (a) Schematic of the experiment. (b) Graph showing relative growth of H1975 tumors. Tumor volume is not significantly different between the two cohorts of mice (t test). (c) Tumor weights (day 30) are not significantly different between the two groups (t test). (d) Total body weights are not significantly different between the two groups. (e) Real-time PCR showing expression of the Nrf2-dependent gene, NQO1, in the liver and tumor tissues.\**P* < 0.05 (t test)**

dependent target gene expression in mice, we measured NQO1 expression in the liver [Figures 2e and 3e]. In addition to the liver, we also measured the induction of Nrf2 signaling in A549 and H1975 tumors. SFN treatment significantly induced the expression of NQO1 in the liver. However, SFN treatment did not upregulate Nrf2 signaling in tumors [Figures 2e and 3e]. These data indicate that systemic SFN treatment induces Nrf2 signaling but does not promote growth of lung cancer xenografts.

### Localized delivery of SFN inhibits the growth of A549 tumors

To determine whether localized delivery of SFN that results in higher accumulation of SFN in the tumor and neighboring

areas can retard the growth of subcutaneous tumors, we selected A549 cells as the model system. A549 cells were injected into the flanks of nude mice, and change in tumor volume was recorded. Once the tumor volumes reached  $50 \pm 20 \text{ mm}^3$ , mice were randomly allocated into 2 groups, and treatment groups were administered SFN ( $1 \mu\text{mol}$ , 5d/wk) by means of subcutaneous injection adjacent to the tumor [Figure 4a]. We monitored the increase in tumor volume with time. The average change in tumor volume was significantly different between the vehicle- and SFN-treated tumors ( $P < 0.05$ ) [Figure 4b]. Tumor weights were significantly higher in the vehicle-treated tumors than in the SFN-treated tumors ( $P = 0.05$ ) [Figure 4c], with no apparent difference in total body weight [Figure 4d] between the two groups.



**Figure 4: Localized delivery of SFN inhibits the growth of A549 lung cancer xenografts. (a)** Schematic of the experiment. **(b)** Graph showing relative growth of A549 tumors treated with 1000 nmol of SFN.  $*P < 0.05$  (t test). **(c)** Tumor weights were significantly lower in the SFN-treated group.  $*P < 0.05$  (t test). **(d)** Body weight did not change significantly in response to SFN treatment. **(e-g)** Relative tumor growth, average tumor weight, and body weight in the cohort of mice treated with 500 nmol of SFN. **(h-j)** Relative expression of Nrf2-dependent genes in the liver and tumor tissues.  $*P < 0.05$ .

Significant inhibition of tumor growth by SFN at a dose of 1  $\mu\text{mol}$  prompted us to determine whether SFN exerts a similar effect at a lower dose (0.5  $\mu\text{mol}$ ) as well. Treatment of A549 xenograft tumors with a lower dose of SFN significantly retarded the growth of A549 tumors in nude mice. Average tumor volume and weight were significantly lower in the SFN-treated cohort of mice [Figures 4e and 4f]. There was no adverse effect of localized SFN treatment on animal health or body weight, although we noticed slight thickening of skin in the area surrounding the injection spot [Figure 4g].

To demonstrate that localized delivery of SFN induced Nrf2-dependent target gene expression, we measured NQO1, HO-1, and GCLC expression in the skin and liver. In addition to these two tissues, we measured the induction of Nrf2 signaling in A549 tumors. SFN treatment significantly induced the expression of HO-1 in the skin with no apparent effect on Nrf2-dependent gene expression in the tumors [Figures 4h–j]. Since SFN was administered locally, we did not notice significant change in Nrf2 dependent gene expression in the liver. These data indicate that localized SFN treatment probably results in greater accumulation of SFN in subcutaneous tumors leading to significant growth inhibition.

## DISCUSSION

Since the early 1980s, naturally occurring antioxidants and vitamins have been investigated and promoted as chemopreventive agents. Antioxidants have been popular as chemopreventive agents because there is strong scientific evidence supporting reduced incidence of epithelial cancers associated with high dietary intake of fruits and vegetables. Because of this evidence, major clinical trials were launched to test  $\beta$ -carotene, alone or in combination with vitamin E or vitamin A.<sup>[41]</sup> The largest trials for chemoprevention of lung cancers in high-risk smoker populations were ATBC, the alpha tocopherol (vitamin E), beta-carotene trial in Finland, and CARET, the beta-carotene and retinol efficacy trial smokers in the United States.<sup>[42]</sup> The results were devastatingly similar, with excess lung cancer incidence and mortality rates in the active treatment arm, compared with those in the placebo arm of each trial.<sup>[41]</sup> The unexpected and unwanted outcomes from the CARET and ATBC trials prompted us to reconsider issues related to both the effectiveness and the safety of micronutrient supplementation and careful evaluation of chemopreventive agents in preclinical cancer models.

SFN derived from broccoli sprouts is currently under

clinical evaluation in diseases in which oxidative stress and inflammation play important roles in disease pathogenesis, namely, pulmonary diseases, such as chronic obstructive pulmonary disease, asthma, and cystic fibrosis, and cardiovascular disease, and protection against radiation dermatitis. In addition to these, SFN is being evaluated as a potential chemopreventive agent in melanoma and breast cancer and as an anticancer agent in patients with recurrent prostate cancer. At present, there are 17 clinical trials listed at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) that are using SFN or an enriched broccoli sprout preparation for treatment of a variety of diseases. The molecular targets of SFN vary depending on cancer stage and target tissue. Although SFN is known to modulate several signaling pathways, it is a potent inducer of Nrf2-Keap1 signaling, and the anticarcinogenic and anti-inflammatory activities of SFN are mediated primarily by Nrf2-dependent induction of phase-II detoxification and antioxidant enzymes.<sup>[13–14, 29–30]</sup>

Results from recent studies suggest that SFN offers protection against tumor development and progression during the post-initiation phase by modulating diverse cellular processes that inhibit the growth of transformed cells.<sup>[14]</sup> The ability of SFN to induce reactive oxygen species production, apoptosis, and cell cycle arrest is associated with regulation of many molecules, including the BCL-2 family of proteins, caspases, p21, cyclins, cyclin-dependent kinases, and histone deacetylases and nuclear factor-kappa-B pathways.<sup>[14]</sup> SFN also suppresses angiogenesis and metastasis by downregulating *hypoxia-inducible factor 1 alpha*, *matrix metalloproteinase 2*, *matrix metalloproteinase 9*, and *vascular endothelial growth factor* in various preclinical models of cancer<sup>[14, 25]</sup> Lastly, SFN inhibits self-renewal of cancer stem cells.<sup>[24, 26]</sup>

However, in the past few years, loss of function mutations in Keap1 and activating mutations in Nrf2 leading to gain of Nrf2 have been reported in lung cancer.<sup>[31, 43]</sup> In addition to lung cancer, gain of Nrf2 function has been reported in other cancers such as prostate, gallbladder, esophagus, breast, skin, and ovarian cancer. In addition to mutations, results from recent studies have demonstrated that the oncogenes K-ras, B-raf, and Myc upregulate Nrf2 signaling to evade senescence and apoptosis and promote tumorigenesis.<sup>[44]</sup>

A comprehensive profiling of SFN bioavailability and tissue distribution kinetics revealed a dose-dependent increase in tissue concentrations after oral administration of SFN in mice.<sup>[45]</sup> SFN accumulation peaked at 2 h in lung, liver, kidney, brain, and plasma, but in small intestine, colon, and prostate, the highest concentrations were recorded at

6 h. Maximum SFN accumulation was detected in small intestine. Furthermore, tissue concentrations of SFN metabolites varied as much as 100-fold between different tissues suggesting that peak plasma concentrations do not always align precisely with target tissues.<sup>[45]</sup> Thus, route of administration may have a critical impact on SFN tissue distribution and accumulation. In this study, we compared three routes of SFN administration; (a) direct delivery to the target organ (lung) using nebulizer, (b) localized delivery to tumor bearing area (subcutaneous injection), and (c) systemic delivery with minimal risk of potential side effects due to systemic distribution (intraperitoneal injection).

To evaluate the effect of prolonged SFN treatment on growth of lung cancer xenografts, we selected two models: (1) A549 cells harboring mutant K-ras oncogene and mutant Keap1 gene<sup>[31]</sup> and (2) H1975 cells harboring mutant EGFR oncogene but wild-type Keap1 and Nrf2. Prolonged systemic SFN treatment did not promote the growth of these tumors *in vivo*. On the contrary, localized delivery of SFN significantly abrogated the growth of Keap1 mutant A549 tumors *in vivo* suggesting that the tumor suppression effects of SFN are mediated by non-Nrf2-dependent signaling mechanisms. Importantly, significant tumor growth inhibition and a trend toward reduction with localized and systemic route of SFN administration, respectively, suggests that localized delivery of SFN probably results in better target tissue distribution and accumulation as compared to the systemic route.

Next, we investigated whether prolonged SFN administration affects oncogenic K-ras (K-ras<sup>G12D</sup>)-driven lung tumorigenesis. A study by DeNicola *et al.*<sup>[36]</sup> showed that oncogenic K-ras signaling upregulates Nrf2 expression and its transcriptional network.<sup>[36]</sup> Interestingly, although SFN administration via aerosol inhalation only modestly affected the Nrf2 signaling, we still noticed a trend toward reduced tumor burden in SFN treated cohort of mice. This raises a possibility that alternative route of administration resulting in localized accumulation and robust induction of Nrf2 signaling may significantly inhibit K-ras mediated lung tumor growth. In summary, SFN treatment does not accelerate K-ras-mediated lung tumorigenesis. However, one limitation of our study is that we only evaluated the effect of SFN on K-ras<sup>G12D</sup>-mediated lung tumorigenesis post oncogenic K-ras activation and tumor foci formation. It remains to be investigated if SFN administration prior to cre-recombinase mediated oncogenic K-ras<sup>LSL-G12D</sup> activation may impact the overall tumor burden.

## CONCLUSIONS

Pharmacological activators of Nrf2 (dithiolethiones, isothiocyanates, and triterpenoids) including SFN have been evaluated as modifiers of multistage carcinogenesis in animal models and no protumorigenic effects have been reported to date. Our data from three preclinical mouse models of lung cancer suggest that prolonged SFN treatment does not promote tumor growth in mice. These findings, along with those from previously published studies on the chemopreventive properties of SFN against chemical carcinogens, support its future clinical development as a drug for chronic obstructive pulmonary disease and other pulmonary diseases.

## ACKNOWLEDGMENT

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