Sulforaphane suppresses the growth of EGFR-overexpressing MDA-MB-468 triple-negative breast cancer cells *in vivo* and *in vitro*

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Abstract. Sulforaphane (SFN), found in cruciferous vegetables, has been found to exert potent antitumor effects against triple-negative breast cancer (TNBC). The present study thus investigated the antitumor activity of SFN against MDA-MB-468 TNBC cells characterized by the overexpression of the epidermal growth factor receptor (EGFR) and the co-deletion of the phosphatase and tensin homolog. SFN exerted concentration-dependent anti-proliferative effects, with a half-maximal inhibitory concentration (IC_{50}) value of 1.8 μM following 72 h of exposure. SFN led to cell cycle arrest at the S phase and more predominantly, at the G2/M phase, and increased the sub-G0/G1 cell population, which represented apoptotic cells, followed by the cleavage of poly(ADP-ribose) polymerase. These data indicate that the inhibitory effects of SFN reflect the combination of S/G2/M cell cycle arrest and apoptotic cell death. SFN upregulated the expression of pro-apoptotic B-cell lymphoma (Bcl)-2-associated X (Bax) without affecting the expression of anti-apoptotic Bcl-2, suggesting that the increased expression of Bax plays a causative role in SFN-induced apoptosis. SFN simultaneously inhibited the activities of protein kinase B (Akt) and mechanistic target of rapamycin (mTOR), thus indicating that SFN substantially inhibited the Akt/mTOR pathway, a survival signaling pathway downstream of the EGFR/phosphatidylinositol 3-kinase. Furthermore, the oral administration of SFN significantly attenuated tumor growth in nude mice transplanted with MDA-MB-468 cells, without any apparent toxicity. On the whole, the findings of the present study suggest that SFN has therapeutic potential for use in the treatment of TNBC with an overactivated signaling pathway downstream of EGFR.

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Introduction

Cancer is an increasing global concern, and breast cancer remains the most frequently diagnosed cancer affecting females, accounting for >20% of all cancer cases in women worldwide (1). Breast cancer is categorized based on cellular markers that reflect the available targeted therapies. Triple-negative breast cancer (TNBC) is a subtype of breast cancer characterized by the suppressed expression levels of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). TNBC accounts for ~10-20% of breast cancer cases and is a highly aggressive disease with frequent early relapses and a very poor overall survival rate (2). Therefore, in addition to its prevention, the development of novel treatment options for this type of breast cancer is crucial due to the limited number of available treatments.

Previous epidemiological studies have suggested that diets rich in cruciferous vegetables, such as broccoli, cabbage and kale reduce the risk of developing a number of common types of cancer, including breast cancer (3,4). Sulforaphane (SFN) is an isothiocyanate derivative generated by the hydrolytic conversion of glucoraphanin, a sulfur-containing compound found in cruciferous vegetables (5). Recently, SFN was shown to be effective in preventing breast cancer at different stages of carcinogenesis by increasing the levels of antioxidants and phase II detoxifying enzymes via the activation of the nuclear factor erythroid 2-related factor 2 (6,7). In addition to its chemopreventive effects, SFN has been found to exert anti-proliferative effects on various human breast cancer cell lines that are representative of a wide range of breast cancer phenotypes by inducing apoptosis, cell cycle arrest and exhibiting anti-angiogenic capacity (6-8). Therefore, SFN may have the potential to prevent and treat all subtypes of breast cancer.

Molecular aberrations in the epidermal growth factor receptor (EGFR)/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) pathway are well-known pathognomonic abnormalities in breast cancer across various subtypes and are commonly observed in TNBC (9). A subset of TNBC (~18%) is known to express EGFR and is associated with a poor prognosis (10). This signaling pathway is also activated in TNBC cells by the stimulation with non-receptor tyrosine kinases, such as the

Src oncoprotein (11), which in turn triggers PI3K activation, followed by the phosphorylation of Akt and mTOR. Moreover, it has been demonstrated that the loss of phosphatase and tensin homolog *(PTEN)*, a tumor suppressor gene that inhibits cell proliferation by inhibiting the PI3K signaling pathway, is a frequent event that occurs in half of TNBC cases (12), and is associated with aggressive behavior and a poor prognosis in patients with TNBC (13). Thus, it is conceivable that the oncogenic activation of the PI3K/Akt/mTOR pathway may be induced in TNBC cells, either by the overexpression/activation of various upstream tyrosine kinases, activating mutations of the PI3K catalytic subunit α, or the loss of function of PTEN. Currently, clinical drugs targeting PI3K/Akt/mTOR signaling have not yet been successfully developed. It has been shown that SFN inhibits the Akt/mTOR pathway, resulting in the decreased survival of phenotypically different breast cancer cells (14). Therefore, SFN may be potentially useful in the treatment of patients with TNBC; however, its precise inhibitory mechanisms remain poorly understood in human TNBC cells presenting an overactivated signaling pathway downstream of EGFR.

Thus, the present study investigated the cellular and molecular mechanisms of the growth-inhibitory activity of SFN against the MDA-MB-468 TNBC cell line exhibiting the activation of the PI3K/Akt/mTOR signaling pathway due to high levels of EGFR expression with the concomitant deletion of PTEN (15,16). In addition, the *in vivo* activity of SFN was examined using a mouse xenograft model in order to determine the potential clinical application of SFN in the prevention and treatment of this type of breast cancer. To the best of our knowledge, the present study is the first to demonstrate the *in vivo* antitumor activity of SFN against MDA-MB-468 TNBC cells overexpressing EGFR with the co-deletion of PTEN.

Materials and methods

Cell culture and chemicals. The present study was performed using the MDA-MB-468 TNBC cells purchased from the American Type Culture Collection (HTB-132) supplied by Summit Pharmaceuticals International Co. The origins of the cell line and its hormone receptor and HER2 status have been previously described (17). The MDA-MB-468 cells lack PTEN repressors (16) and possess high EGFR levels (15). This cell line was cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (FBS; Cosmo Bio Co., Ltd.), 100 IU/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. SFN for use in the *in vitro* experiments was purchased from Sigma-Aldrich; Merck KGaA and stored at -20˚C. A 225 mM stock solution was prepared by dissolving the original SFN with dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) and diluted with RPMI-1640 immediately prior to experimental use. The final concentration of DMSO for all experiments and treatments (including vehicle controls, where no SFN was added) was maintained at ≤0.002%. These concentrations of DMSO were confirmed to be non-cytotoxic for at least 72 h of consecutive treatment (data not shown).

Determination of growth inhibition. The anti-proliferative effects of SFN on the growth of MDA-MB-468 cells were assessed using the Cell Counting Kit-8 (CCK-8; Dōjindo Laboratories, Inc.) according to the manufacturer's protocol. Briefly, 2,000 cells/100 μl suspension were seeded into each well of a 96-well plate (Corning Inc.). Following 24 h of incubation, 100 μl SFN at various concentrations (0-4.5 μM) was added and cells were further cultured for up to 72 h. The culture medium was then replaced with CCK-8 solution, and incubated for 2 h at 37˚C. The relative number of viable cells was determined by comparing the absorbance (490 nm) of the treated cells with the corresponding absorbance of vehicle-treated cells taken as 100%, using Infinite 200 Pro, Tecan Trading AG. The IC_{50} value was defined as the concentration at which cell viability was inhibited by 50%.

Cell cycle analysis and measurement of apoptosis. At different time points (0, 24, 48 h) following treatment with 2 μM SFN (an approximate IC_{50} concentration), floating and trypsinized adherent cells were combined, fixed in 70% ethanol for 2 h at 4˚C and stored at 4˚C prior to use in cell cycle analysis. Following the removal of ethanol by centrifugation at 500 x g for 5 min in 4˚C, the cells were washed with PBS and stained with a solution containing RNase A (10 μ1/ml) and propidium iodide (PI; 50 μg/ml; Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Cell cycle analyses were performed on a Gallios flow cytometer with Kaluza ver. 1.2 software (Beckman Coulter, Inc.). Each cell cycle phase was classified based on each histogram, and the percentages were calculated. The extent of apoptosis was determined by measuring the sub G0/G1 population detected by flow cytometry in the same manner as described above.

Western blot analysis of signaling proteins involved in cell growth and apoptosis. Following treatment with 2 μM SFN, the cells were washed with ice-cold PBS and scraped into 0.5 ml lysis buffer (Bio-Rad Laboratories, Inc.). Then protein concentration was determined using the Bradford method. Proteins (10 μg/lane) were resolved by 4-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories, Inc.) and electrotransferred onto a polyvinylidene difluoride membrane (GE Healthcare; Cytiva). Non-specific binding sites were blocked by incubating the membranes in blocking buffer (Nacalai Tesque, Inc.) at room temperature for 30 min. The membranes were then incubated overnight at 4˚C with primary antibodies against Akt (cat. no. 9272; 1:200), phosphorylated (p-)Akt (Ser473; cat. no. 4058; 1:200), mTOR (cat. no. 2983; 1:2,000), p-mTOR (Ser2448; cat. no. 2971; 1:2,000), B-cell lymphoma (Bcl)-2 (cat. no. 2876; 1:1,000), Bcl-2-associated X (Bax; cat. no. 2772; 1:1,000) or β-actin (cat. no. 4967; 1:500) (all from Cell Signaling Technology, Inc.). The membranes were hybridized with horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; 1:1,000, Cell Signaling Technology, Inc.) for 1 h at room temperature. Immunoblots were developed using enhanced chemiluminescence system (GE Healthcare; Cytiva) and quantified using a Fusion Fx Imaging System (Vilber). The density ratios are shown at the bottom of the bands (in graphs) as a relative ratio vs. the untreated control.

Apoptosis was assessed by poly (ADP-ribose) polymerase (PARP) cleavage detected using western blot analysis with PARP antibody (cat. no. 9542; 1:1,000, Cell Signaling Technology, Inc.) using the aforementioned conditions. PARP is a substrate for certain caspases that are activated during the early stages of apoptosis. These proteases cleave PARP to fragments of ~89 and 24 kDa. The detection of the 89 kDa PARP fragment with anti-PARP serves as an early marker of apoptosis.

In vivo tumor xenograft model. All animal procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the Nakamura Gakuen University (Approval no. 2018-1). Athymic nude mice (BALB/cAJcl-nu/nu) were obtained from CLEA Japan, Inc. and housed at the Nakamura-Gakuen Animal Center under the following conditions: A temperature of 24˚C, 40% humidity and a 12-h-light/-dark cycle, with free access to food and water.

A xenograft model of human TNBC was established by the subcutaneous dorsal flank injections of MDA-MB-468 cells (~2.5x10^6) into 8 female nude mice (4 weeks of age). At 1 week prior to implantation, the 8 female nude mice (4 weeks of age) were divided into two groups, each consisting of 4 mice. A 100 μl SFN (LKT Laboratories, Inc.) solution or PBS (vehicle control) were orally administered daily using polyurethane tubes (FCR&Bio Co., Ltd.) from 1 week prior to the inoculation of the tumor cells to the end of the experiment. The vehicle control group was treated with 100 μl PBS, and the SFN group with 100 μl of 1 mM SFN solution prepared by dissolving SFN with PBS immediately prior to administration. This oral concentration of SFN (1 mM) was arbitrarily determined by preliminary experiments (data not shown). Initially, 6 μM SFN were applied daily by referring to two previous *in vivo* experiments that used 5.6-6.0 μM SFN from LKT Laboratories, Inc. (18,19). The concentration of SFN administered daily was gradually increased until effects on tumor growth were observed, and finally found that 1 mM SFN was a sufficient dose for attenuating tumor growth (17.7 μg SFN/mouse/day). Food intake and body weight were monitored during the experiment. Tumor size was measured every week using calipers, and tumor volume was calculated using the following formula: Tumor volume (mm^3)=[length (mm)] x[width (mm)] 2x0.52. At the end of the study period (7 weeks following tumor cell implantation), the mice were anesthetized using an intraperitoneal injection of pentobarbital (75 mg/kg) followed by euthanasia via exsanguination, and the tumors were removed, weighed and processed for pathological analysis.

Hematoxylin and eosin (H&E) staining. The tumors were fixed in 10% buffered formalin solution at least for 24 h at room temperature until paraffin embedding. Subsequently, the paraffin-embedded tissue blocks were cut into 3-μm-thick sections. The sections were then stained with H&E (FUJI FILM Wako Pure Chemical Corporation) each for 10 min at room temperature. Histopathological images were obtained using an Olympus FSX100 all-in-one inverted microscope (Olympus Corporation).

Statistical analyses. Statistical analyses were performed using statistical software (IBM SPSS Statistics version 25). Data from at least three independent experiments performed in triplicate are presented as the mean ± standard deviation (SD). ANOVA was used to compare changes over time in cytotoxicity experiments After having tested for normality, ANOVA was used for parametric data, and the Mann-Whitney U test for non-parametric data. Comparisons among multiple groups were first performed using one-way ANOVA. If the results revealed significant differences, comparisons were performed using Dunnett's t-test. The Mann-Whitney U test was used to compare two groups, the SF-treated group and the non-treated group, in animal experiments. Statistical tests were two-tailed, and a P-value <0.05 was considered to indicate a statistically significant difference.

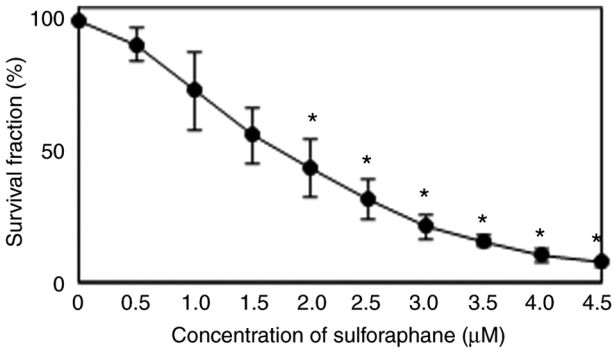
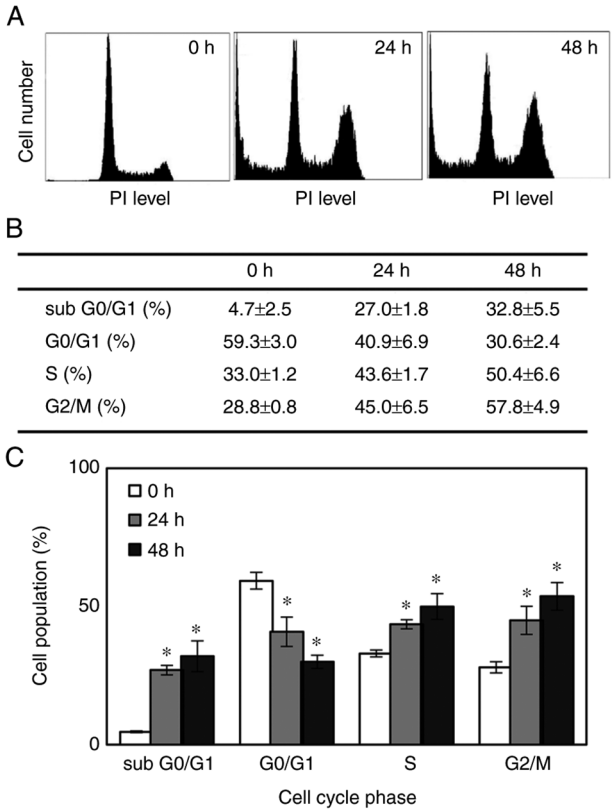


Figure 1. Effect of sulforaphane on the growth of the MDA-MB-468 triple-negative breast cancer cell line. Cells were incubated with various concentrations of sulforaphane for 72 h. The vertical bars represent the mean ± standard deviation (SD) of three independent experiments. *P<0.05, significant difference vs. the control (0 h).

Results

Effects of SFN on cell proliferation and survival. To determine the effects of SFN on cell growth and survival, MDA-MB-468 cells were treated with various concentrations of SFN for 72 h. As shown in Fig. 1, SFN exhibited concentration-dependent antitumor activity against the MDA-MB-468 cells. The 50% inhibitory concentration (IC_{50}) was 1.8±0.4 μM following 72 h of exposure.

Time-course analysis of the effects of SFN on cell cycle progression and apoptosis. To examine whether the inhibitory effects observed in the cytotoxicity assays reflect the arrest or delay of cell cycle progression or apoptotic cell death, the cells were treated with 2 μM SFN, and cell cycle progression and apoptosis were evaluated by fluorescence-activated cell sorting (FACS) analysis. Representative cell cycle distributions following consecutive treatment with SFN at the indicated time points are shown in Fig. 2A. When the MDA-MB-468 cells were treated with 2 μM SFN, the proportion of cells in the S and G2/M phases significantly increased from 33 and 28.8% at the beginning of the treatment to 43.6 and 45.0%, respectively, with a corresponding decrease in the number of cells in the G0/G1 phase following 24 h of exposure. The percentage of cells in each cell cycle phase was not significantly altered after 48 h consecutive exposure compared with the 24-h time point (Fig. 2B and C). Therefore, SFN arrested the cell cycle at the S phase and more predominantly at the G2/M phase.



	0 h	24 h	48 h
sub G0/G1 (%)	4.7±2.5	27.0±1.8	32.8±5.5
G0/G1 (%)	59.3±3.0	40.9±6.9	30.6±2.4
S (%)	33.0±1.2	43.6±1.7	50.4±6.6
G2/M (%)	28.8±0.8	45.0±6.5	57.8±4.9

Figure 2. Time-course analyses of the effects of SFN on MDA-MB-468 cell cycle progression and apoptosis determined using flow cytometry. Cells were treated with 2 μM SFN and subjected to fluorescence-activated cell sorting analysis. (A) Representative cell cycle distributions following treatment with SFN for the indicated periods of time. The apoptotic populations were determined by measuring the cells in the sub-G1 phase. (B) Percentages of the total cell population in the different phases of cell cycle following treatment with SFN for the indicated periods of time. The values represent the mean ± SD of three independent experiments. (C) Percentages of the total cell population in the different phases of the cell cycle shown in panel B are depicted as column charts. The vertical bars indicate the mean ± SD of three independent experiments. *P<0.05, significant difference vs. the control (0 h). SFN, sulforaphane.

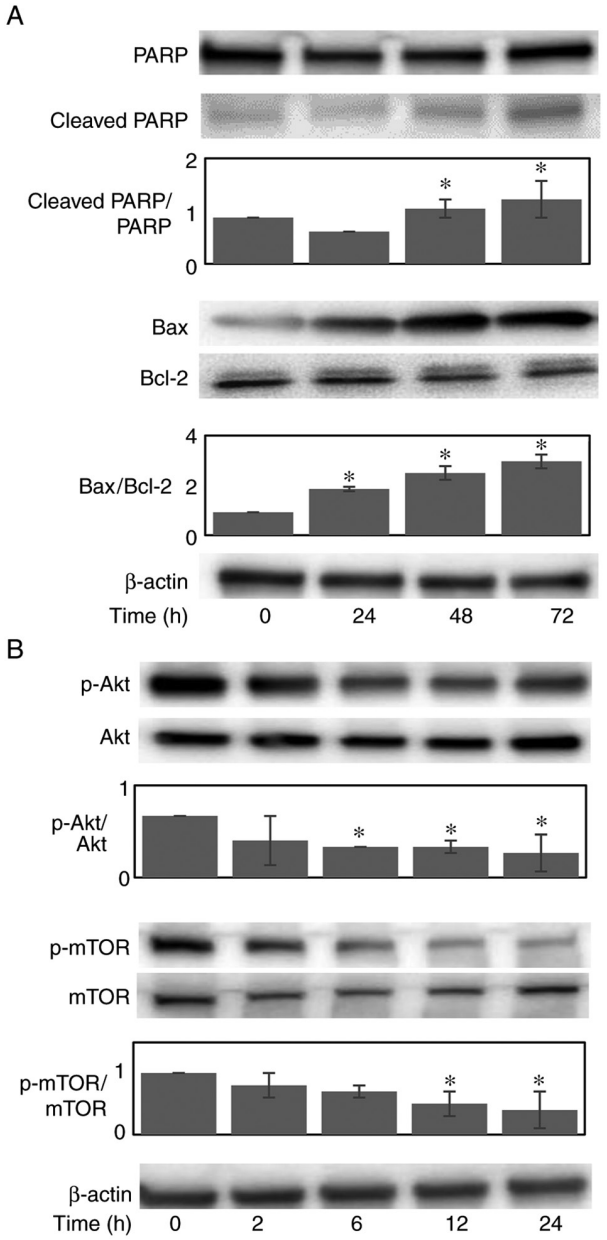


Figure 3. Effect of SFN on the activation of signaling molecules for cell proliferation and apoptosis/survival. Cells were treated with 2 μM SFN for the indicated periods of time and harvested for western blot analyses. (A) Representative western blots for the effects of SFN on apoptotic Bax, anti-apoptotic Bcl-2 and PARP. (B) Representative western blots are shown for total and phosphorylated Akt, p-Akt (ser473), mTOR and p-mTOR (ser2448). β-actin was used as the internal control. The column bars of cleaved PARP/PARP, Bax/Bcl-2, p-Akt/Akt, and p-mTOR/mTOR ratios at the indicated time points are also shown. The vertical bars indicate the mean expression level ± SD of three independent experiments. *P<0.05, significant difference vs. the control (0 h). SFN, sulforaphane; Bax, B-cell lymphoma (Bcl)-2-associated X; Bcl-2, B-cell lymphoma-2; Akt, protein kinase B; mTOR, mechanistic target of rapamycin; PARP, poly(ADP-ribose) polymerase; p-, phosphorylated.

The sub G0/G1 cell population, which represents apoptotic cells, abruptly increased from 4.7 to 27.0 and 32.8% following 24 and 48 h of exposure, respectively (Fig. 2B and C), increasing by almost 7-fold following 48 h of exposure. Furthermore, the cleavage of PARP, which serves as an early marker of apoptosis, was demonstrated at 48 h post-treatment (Fig. 3A). The dissociation between the appearance of the initiation of the apoptotic cellular event (cell population at the sub G0/G1 phase) and the cleavage of PARP may be explained by the difference in their detection times. These data indicated that the observed SFN-induced growth decline appeared to be due to the combined effects of the progressive expansion of the apoptotic cell population and the S/G2/M arrest of the cell cycle.

Effects of SFN on the expression of pro- and anti-apoptotic proteins. To clarify the apoptotic mechanisms induced by SFN, the protein expression levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax were examined (Fig. 3A). Upon treatment with 2 μM SFN, the expression of Bax was increased in a time-dependent manner, whereas the protein expression of Bcl-2 remained unaltered. Thus, the Bax/Bcl-2 ratio increased up to 3.2-fold following 72 h of consecutive treatment. These results suggest that the increased expression of Bax plays a causative role in SFN-induced apoptosis.

Effects of SFN on the activation of Akt/mTOR signaling molecules. Since the activation of PI3K/Akt/mTOR, a major signaling pathway involved in cell proliferation and survival,

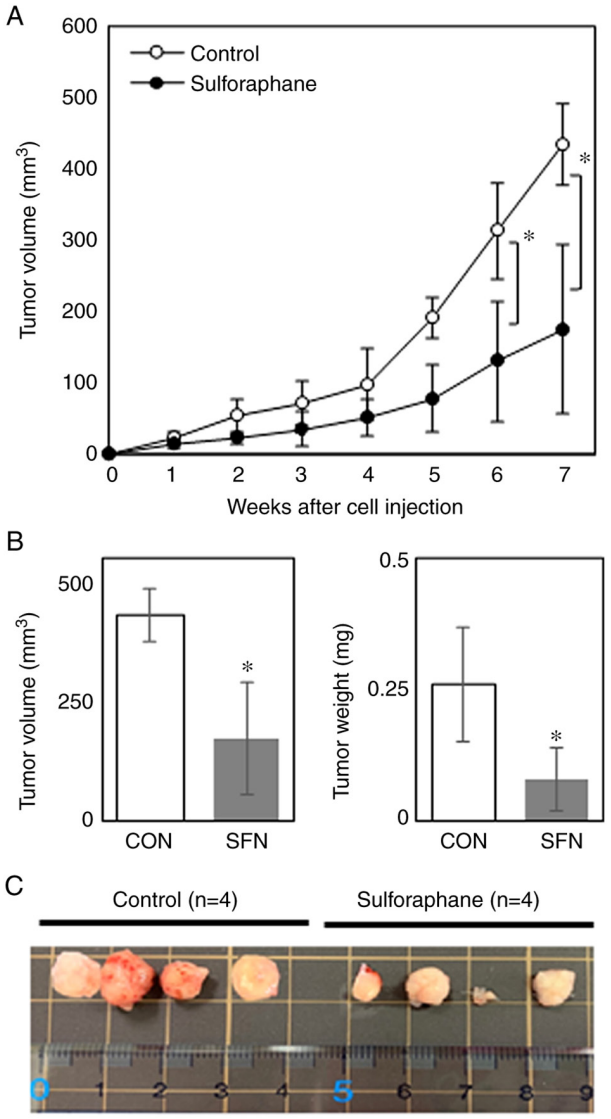


Figure 4. Effects of SFN on tumors transplanted from MDA-MB-468 cells into nude mice. The experimental treatment protocol is described in the 'Materials and methods section'. (A) Time-course changes in the mean tumor volume in the untreated control and SFN-treated mice implanted with MDA-MB-468 cells. Four samples were analyzed in each group. The vertical bars indicate the mean tumor volume ± SD. (B) Column charts indicate the volume and weight of animal tumors excised and measured at the end of the experiment. The vertical bars indicate the mean tumor volume ± SD. (C) Images of xenograft tumors excised from four individual mice in each group at the end of the experiment. SFN, sulforaphane. *P<0.05, significant difference vs. control (untreated).

is considered to be activated in MDA-MB-468 TNBC cells due to their biological features, including the overexpression of EGFR and the co-deletion of PTEN (15,16), the present study examined the effects of SFN on the expression and activation (phosphorylation) of these proteins. Upon treatment with 2 μM SFN, the phosphorylation of Akt and mTOR was substantially inhibited in a time-dependent manner (Fig. 3B). These data thus indicated that the SFN-induced reduction in cell proliferation and survival appeared to be mediated by the inactivation of Akt and mTOR.

Effects of SFN on MDA-MB-468 cells xenotransplanted into nude mice. An *in vivo* experiment was conducted using nude mice xenotransplanted with MDA-MB-468 cells to determine whether the inhibitory effects of SFN on tumor development are observed when administered orally to mice. Although the tumors grew rapidly at ~5 weeks following cell inoculation in control mice, the oral administration of SFN significantly suppressed tumor growth from 5 weeks following inoculation (Fig. 4A), reducing the tumor size and tumor weight by ~60 and 70%, respectively, compared with the untreated control mice at 7 weeks following cell inoculation (Fig. 4B). Images of the xenograft tumors excised from four individual mice in each group at the end of experiment are depicted in Fig. 4C. Although individual tumors varied in size, the tumor sizes appeared to be evidently smaller in the SFN-treated group compared with the control group. Representative images of tumor tissue sections stained with H&E are presented in Fig. 5. The tumor specimens from SFN-treated mice exhibited a degenerated tumor cell appearance with pyknotic nuclei, resembling apoptotic cells (Fig. 5). Furthermore, no significant differences were observed in body weight and food consumption between the two groups (Table I), suggesting that SFN did not exert any or minimal adverse effects at the oral concentration used in the present study. These data thus indicated that SFN substantially inhibited TNBC tumor growth *in vivo* without exerting any apparent toxic effects.

Discussion

SFN is widely recognized as a promising chemopreventive agent with effects against numerous types of human cancers through a variety of mechanisms (20). Moreover, SFN has been reported to potentially prevent breast cancer development and recurrence (7). In the present study, it was found that SFN inhibited the proliferation of MDA-MB-468 TNBC cells with an IC_{50} value of ~2 μM following 72 h of exposure. This IC_{50} value appears to be quite low compared with those of various phytochemicals tested in the authors' laboratory for TNBC (21-24), indicating that SFN is a promising candidate for preventing TNBC among various phytochemicals.

Moreover, the present *in vivo* experiment using nude mice xenotransplanted with MDA-MB-468 cells revealed that the *per os* administration of SFN evidently attenuated tumor growth over a period of 7 weeks, reducing the tumor size by 60% compared with the control. Adverse effects to major organs were considered negligible as there were no significant differences in body weight gain and the consumption of food between the two groups, although this is indirect evidence of the adverse effects. More precisely, the examinations of hematological and biochemical toxicities are required in nude mice. A similar result has been reported using MDA-MB-231 TNBC xenografts (25); however, to the best of our knowledge, the present study is the first to demonstrate the *in vivo* antitumor activity of SFN against MDA-MB-468 cells. Moreover, SFN has been reported to synergistically enhance the efficacy of several anticancer drugs, including cisplatin (26), doxorubicin (25,27) and paclitaxel (28) in various types of human cancer cells. Therefore, co-treatment with chemotherapeutic agents and SFN may reduce the administered doses, thereby alleviating the adverse effects of anticancer agents. Furthermore, it has been reported that human subjects who ingested 100 g of broccoli daily as a soup exhibited a peak

Table I. Body weight and total food consumption of mice xenotransplanted with MDA-MB-468 cells.

Parameter	Control (n=4)	Sulforaphane (n=4)	P-value[a]
Body weight[b] (g)	22.5±1.82	23.5±1.61	0.468
Food intake[c] (g)	210.4±26.72	213.3±27.41	0.356

Date are presented as the mean ± SD. [a]Data were analyzed using the Mann-Whitney U-test and values indicate comparisons vs. the control (untreated) group. [b]Determined at 7 weeks following cell injection. [c]Total food consumption during the experiment.

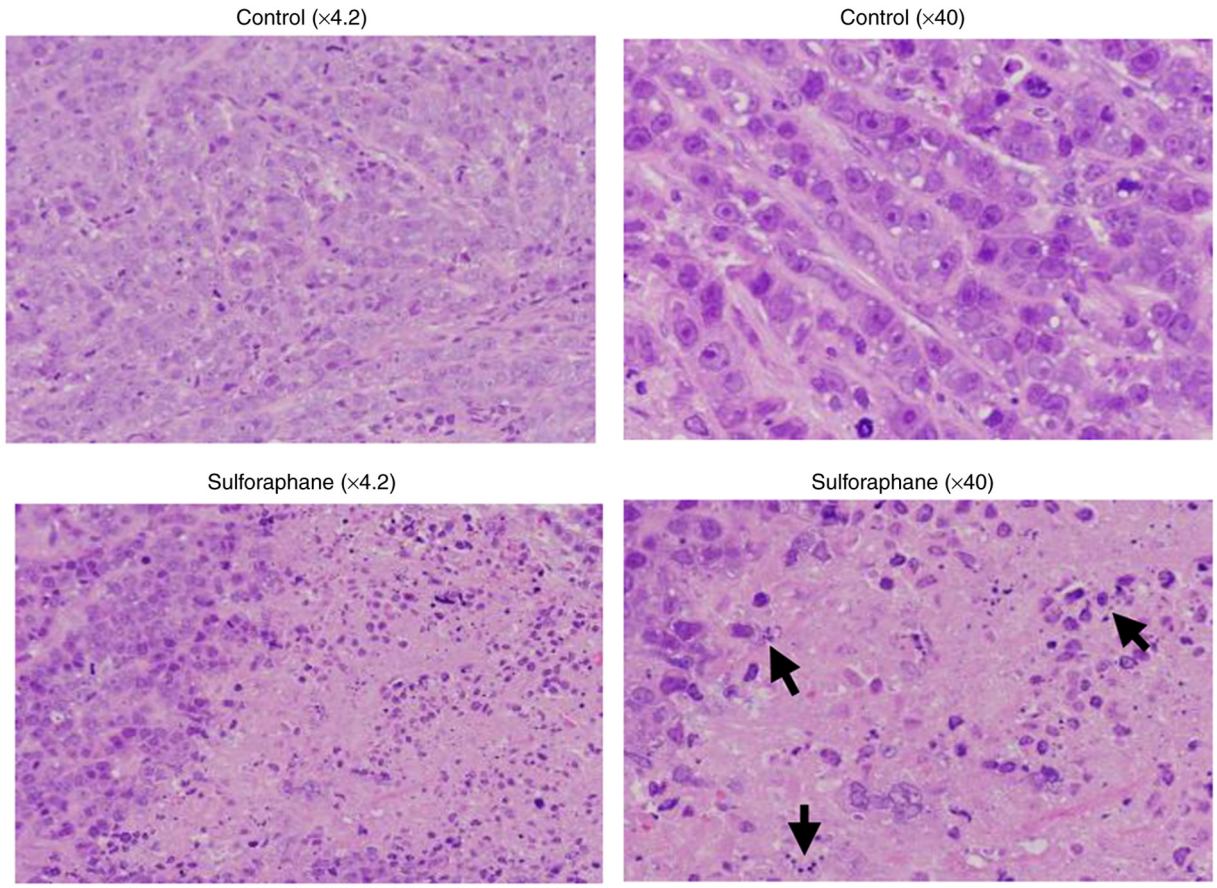


Figure 5. Mice were sacrificed on day 35, and tumors were fixed with 10% formalin, sectioned, and stained with hematoxylin and eosin to examine the tumor cell morphology. Arrowheads indicate pyknotic nuclei that are small condensed nuclei from apoptotic cells.

plasma concentration of ~2-7 μM SFN metabolites, including free SFN (29). Thus, it is conceivable that consuming a diet rich in cruciferous vegetables, such as broccoli sprouts, may reduce the risk and development of TNBC and may potentially be useful for the treatment of TNBC.

Cell cycle checkpoints are crucial for controlling the mechanisms that ensure the proper execution of cell cycle events. SFN has been shown to modulate cell cycle progression in several cellular models, such as prostate, colon, breast and bladder cancers, arresting cells in the G1 (30,31) or the G2/M phase (32-34), depending on the cell type, the treatment concentration and the duration of exposure (35). In the present study, SFN inhibited the proliferation of MDA-MB-468 TNBC cells by inducing S/G2/M cell cycle arrest, causing a blockade of cell cycle entry into mitosis, as previously shown in different TNBC cell lines, including MDA-MB-231 (36). Furthermore, the sub G0/G1 cell population, which represents apoptotic cells, increased, followed by the cleavage of PARP, which serves as a marker of cells undergoing apoptosis. Therefore, these data indicate that the inhibitory effects of SFN observed in cytotoxicity assays reflect the combination of SFN-induced S/G2/M cell cycle arrest and apoptotic cell death.

Although SFN has been found to induce the apoptosis of a variety of breast cancer cells, the mechanisms through which SFN induces apoptosis varies between different cells. The present study demonstrated that SFN upregulated the protein expression of pro-apoptotic Bax, which has been shown to induce apoptosis by promoting the release of cytochrome *c*, as a result of its translocation from the cytosol to the

mitochondria (37). SFN has also been reported to induce the downregulation of anti-apoptotic Bcl-2 protein in several breast cancer cell lines (36). A previous study using MDA-MB-468 cells reported that Bcl-2 levels decreased in a concentration-dependent manner from 5 μM (36). In the present study, however, Bcl-2 expression was not altered. This may be due to the concentration that we used for the experiment in which the effect of SFN on apoptosis signaling proteins was evaluated at a concentration of 2 μM. Nonetheless, the resultant increase in the Bax/Bcl-2 ratio may play an important role in SFN-induced apoptosis in MDA-MB-468 cells at such a low level of SFN.

Studies on the mechanisms underlying the anticancer activities of SFN have indicated that its regulatory effects on the tumor cell cycle, apoptosis and angiogenesis are mediated by the modulation of the related signaling pathways (6-8). MDA-MB-468 TNBC cells are devoid of PTEN, which antagonizes the activity of PI3K, and exhibit high levels of EGFR (15,16), which functions upstream of PI3K. Akt plays a critical role in controlling survival by directly phosphorylating mTOR at Ser2448 (38), leading to an increase in downstream molecules (39). Upon treatment of the MDA-MB-468 cells with SFN, Akt activity was inhibited with a simultaneous decrease in mTOR activity, indicating that SFN substantially inhibited the PI3K/Akt/mTOR pathway even in cells with the overactivation of the downstream pathway caused by the overexpression of EGFR and co-deletion of PTEN. Since EGFR overexpression and loss of PTEN are frequently occurring events in TNBC cases, and are associated with aggressive behavior and a poor prognosis of patients with TNBC (10,12,13), SFN appears to be a promising target drug acting against survival signaling downstream of PI3K in these patients. Moreover, such activities of SFN appear to be crucial as mitogenic and anti-apoptotic potentials are driven by the activation of intracellular signaling molecules upstream of PI3K in almost all cancer cells.

Despite recent advances in breast cancer treatment, breast cancer recurrence is a major problem and the principal cause of breast cancer-related deaths. Emerging evidence suggests the existence of cancer stem cells, a population of cells capable of self-renewal and initiating tumor growth, which might be responsible for breast cancer recurrence (40). Recently, SFN has gained immense attention due to its wide safety profile and ability to target heterogeneous populations of cancer cells, including cancer stem cells. Accordingly, SFN has been shown to reduce the tumor volume in TNBC stem-like cells (MDA-MB-231-Luc-D3H1 cell line) administered daily by intraperitoneal injection (41). Moreover, Burnett *et al* (42) reported that the intraperitoneal injection of SFN enhanced the anticancer activity of taxanes against TNBC by killing cancer stem cells. In the present study, the oral administration of SFN inhibited the growth of xenotransplanted MDA-MB-468 TNBC tumors consisting of a population of *in vivo* selected and thus highly tumorigenic cells resembling cancer stem cells. The major difference between the two aforementioned previous studies and the present study is the design of the administration route of SFN. The present experimental design mimics the ordinary method of SFN uptake included in dietary vegetables. Therefore, absorption routes via either the intestine or peritoneal membrane may greatly affect the pharmacokinetics of SFN administered via either route. To the best of our knowledge, the present study is also the first to demonstrate the *in vivo* antitumor activity of SFN against the MDA-MB-468 TNBC cell line exhibiting the overactivation of the signaling pathway downstream of EGFR due to EGFR overexpression and the deletion of PTEN, as opposed to SUM149 cells, which possess tumor suppressor BRCA1 mutation (43).

In conclusion, the data of the present study suggest that SFN may prove to be potentially useful, not only for the prevention and treatment, but also for the reduction of the recurrence of TNBC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AY, MO and SN designed the study. AY and MO conducted the research. AY, MO, and MT analyzed the data. MO and SN wrote the manuscript. All authors had the primary responsibility for the final content, and read and approved the final manuscript. All authors confirmed the authenticity of the raw data.

Ethics approval and consent to participate

All animal procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the Nakamura Gakuen University (no. 2018-1).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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