



## Cauliflower bioactive compound sulforaphane inhibits breast cancer development by suppressing NF- $\kappa$ B /MMP-9 signaling pathway expression

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### ARTICLE INFO

#### Original paper

#### Article history:

Received: January 11, 2022

Accepted: April 09, 2022

Published: April 30, 2022

#### Keywords:

cauliflower, sulforaphane, breast cancer, NF- $\kappa$ B, MMP-9

### ABSTRACT

In recent years, anti-cancer plant food development and research have received increasing attention, and cauliflower is one of the vegetables with anti-cancer effects. Sulforaphane (SFN) is one of the main anti-cancer components in cauliflower. In this study, the mechanism of action of SFN in anti-breast cancer was investigated using SFN, a bioactive compound extracted from cauliflower. For this purpose, SFN was extracted from cauliflower using rotary evaporation and silica gel chromatography, and the extracted SFN was used for in vitro and in vivo experiments. Breast cancer cells MCF-7, MDA-MB-231 and MDA-MB-231 xenograft tumor model mice were treated with SFN, pcDNA3.1-MMP-9, Si-RNA-MMP-9 and Si-RNA-NF- $\kappa$ B, respectively, and the corresponding saline treatment or blank plasmid treatment was used as control. The gene expression of NF- $\kappa$ B and MMP-9 in each group was detected by RT-PCR, and the protein phosphorylation level of MMP-9 was measured by Western blotting assay. WST 1 assay, MTT assay and flow cytometric analysis were used to detect the activity, proliferation and apoptosis levels of breast cancer cells. The tumor histopathology of the xenograft tumor model mice after SFN treatment was examined by HE staining. Results showed that Breast cancer cells treated with SFN showed reduced cell proliferation, decreased cell activity, increased apoptosis ratio, and inhibited gene expression and protein phosphorylation of MMP-9 as well as gene expression of NF- $\kappa$ B ( $P < 0.05$ ). The same effect occurred with transfection of Si-RNA-MMP-9 and Si-RNA-NF- $\kappa$ B in breast cancer cells, while transfection of pcDNA3.1-MMP-9 plasmid significantly redeemed the inhibitory effect of SFN on breast cancer cells ( $P < 0.05$ ). MDA-MB-231 xenograft tumor model mice treated with SFN showed significant improvement in the pathological condition of the tumor tissue. Then, SFN may inhibit breast cancer development by regulating the NF- $\kappa$ B /MMP-9 signaling pathway.

DOI: <http://dx.doi.org/10.14715/cmb/2022.68.4.17> Copyright: © 2022 by the C.M.B. Association. All rights reserved.



### Introduction

Breast cancer is one of the leading causes of death from malignancy in women (1,2). Metastases cause most breast cancer deaths from the primary tumor site to distant sites (2). Despite successful treatment of the primary malignancy, tumor cells can recur in other parts of the body through blood or lymphatic channels, followed by metastatic spread (2). This leads to distant metastasis of the patient's tumor areas, including bone, lung, liver, kidney, thyroid and brain (2).

Epidemiological studies have consistently shown that increased dietary intake of fruits and vegetables is strongly associated with a reduced risk of developing chronic diseases such as cardiovascular disease, diabetes and cancer (3,4). Long-term intake treatment with anti-cancer plant foods is effective in reducing the patient's illness and recurrence rate of cancer

compared to traditional methods of cancer treatment (5,6). Cauliflower, also known as broccoli, brassica, and tender stem cauliflower, contains high levels of sulforaphane (SFN) (7). SFN has been shown to have anticancer properties against a variety of cancer cell lines (8-10). SFN was first identified as a potent inducer of phase 2 detoxification enzymes, and subsequent studies have identified other anticancer and antioxidant mechanisms, including induction of cystatin protease and glutathione S-transferase, inhibition of cytochrome P450 isozymes, and reduction of the DNA-binding capacity of NF- $\kappa$ B (11,12). The aberrant activation of the transcription factor NF- $\kappa$ B is strongly associated with inflammatory, autoimmune diseases and cancer (13). Moreover, NF- $\kappa$ B is also able to regulate the expression of several genes and plays a key role in cell proliferation, immune and stress responses (13).

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Cellular and Molecular Biology, 2022, 68(4): 134-143

Matrix metalloproteinase-9 (MMP-9) is produced by mesenchymal, epithelial and hematopoietic cells. It is also generated by different types of tumor cells and is one of the most complex MMPs, belonging to the gelatinase family (14). It is also a downstream gene of NF- $\kappa$ B, regulated by NF- $\kappa$ B and involved in tumor metastasis and various inflammatory and pathological processes (15,16). The expression and repression of the MMP-9 gene can be involved in the development of various diseases such as trophoblast implantation, bone resorption, inflammation and arthritis (16,17). In recent years, there have been more studies on the role of MMP-9 in regulating the invasion and activity of tumor cells (17).

Although there have been many studies on cancer, the molecular mechanism of cancer inhibition by SFN is still unclear. To investigate the molecular mechanism of SFN in breast cancer inhibition, we used human breast cancer cells and nude mice for relevant molecular experiments to verify the correlation between SFN and breast cancer inhibition. We demonstrated that SFN inhibited breast cancer development by suppressing NF- $\kappa$ B and thus MMP-9 expression and activity in vitro. These data suggest that SFN may inhibit breast cancer development and progression by modulating the NF- $\kappa$ B/ MMP-9 signaling pathway, with promising results for the treatment of breast cancer.

## Materials and Methods

### Extraction of SFN

SFN was extracted from fresh cauliflower by rotary evaporation concentration. Briefly, the cauliflower was dehydrated in a drying environment at 100°C. The dried cauliflower was subsequently ground and filtered to remove the impurities. The 8g of filtered product was measured and placed in a triangular flask. Then, the enzyme liquid in the newly collected mustard seeds was extracted using an ultrasonic crushing method, and the enzyme solution in the extracted mustard seeds was added to a triangular flask containing dried cauliflower powder and subjected to enzymatic digestion at room temperature for 60 min. The mixture was then freeze-dried, added with 300 mL of 95% ethanol, extracted by crushing using ultrasound for 1 h, left to stand for 30 min and then filtered and compressed. It was extracted twice by adding ethyl acetate (E809173, Maclean's,

Shanghai, China) at a volume ratio of 1:5 and then concentrated at 70°C. Methanol was fixed to 30 mL. It was degreased, followed by a silica gel chromatography column and elution treatment at a flow rate of 15~25 mL/min. The different tube fractions collected were determined by TLC (Thin Layer Chromatography). The SFN-containing fractions were combined together and the purity of the extracted SFN was analyzed by HPLC with the standard SFN (BP1347, Preferred Method, Chengdu, China).

### Cell culture and transfection

Breast cancer cell lines MCF-7 (PT-1488) and MDA-MB-231 (HTB-26) were purchased from ATCC Biotechnology, Inc. Both MCF-7 and MDA-MB-231 cell lines were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, USA) with 10% fetal bovine serum (FBS), 0.5% chicken embryo extract, 1% penicillin and streptomycin. The incubation conditions were 37°C and 5% CO<sub>2</sub>. The cells in the SFN-treated group (SFN) were then treated with 100, 200, and 400 nmol/L of SFN in a normal medium. The Control group was then studied with the corresponding MCF-7 or MDA-MB-231 cell lines. Si-RNA-MMP-9 (Forward: 5'-CAUCACCUAUUGGAUCCAA-3', Reverse: 5'-UUGGAUCCAAUAGGUGAUG-3') and Si-RNA-NF- $\kappa$ B (Forward: 5'-AAACUCAUCAUAGUUGAUGGUGCUC-3', Reverse: 5'-GAGCACCAUCAACUAUGAUGAGUUUU-3') were synthesized by Kingsray Biologicals (Nanjing, China) and the cells were transfected by Lipofectamine 3000 (L3000150, Sigma, USA) kit. The control group underwent the same operation to transfect cells with Si-RNA-Normal control (Si-RNA-NC). The pcDNA3.1-MMP-9 overexpression plasmid and the control plasmid pcDNA3.1-Normal control were synthesized and constructed by Kingsray Biologicals (Nanjing, China). The 50  $\mu$ L of receptor cells were taken and added to 1.5 ml EP tubes with an ice bath. Then, 5  $\mu$ L of ligated plasmid mix (plasmid containing ampicillin expression gene) was added on ice for 15 min. In addition, 10 mL of LB medium (containing ampicillin) was added and shaken for 1 hour at 37°C. The 200 $\mu$ L of the above culture solution was taken and dropped into LB plate culture dishes

containing ampicillin. The colonies were selected for monoclonal culture, and then plasmid extraction was carried out with QIAGEN (Germany) plasmid extraction kit. The 5  $\mu$ g of extracted plasmid was mixed with optimized medium opti-mem 200  $\mu$ L and transfection solution 10  $\mu$ L. Then it was added to an SFN cell medium containing 200nmol/L. pcDNA3.1-Normal control was expressed as SFN.

### Construction of xenograft tumor model mice

MDA-MB-231 cells (1 x 10<sup>7</sup> cells/each) were injected subcutaneously into the right dorsum of female nude mice (Southern Medical University Laboratory Animal Management Center, Guangzhou, China). When the tumors reached approximately 50 mm<sup>2</sup>, the mice were randomly divided into two groups (n=6). The control group was treated with saline in 2% DMSO / 8% olive oil, while the SFN group was treated with extracted SFN at 50 mg/kg every three days. Also, the tumor size was measured using the following formula: 0.5 x length x width<sup>2</sup>. After 21 days of treatment, the mice were executed, and the tumors were collected and weighed. A portion of each tumor was fixed with 4% paraformaldehyde, and the rest of the tumor was frozen with liquid nitrogen. All animal experiments were performed in accordance with protocols approved by the Affiliated People's Hospital of Ningbo University Animal Research Regulatory Committee and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from cells or tumor tissue using RNAiso Plus reagent (TaKaRa). The RNA was reverse transcribed to cDNA according to the instructions for the PrimeScript RT kit (TaKaRa). The relative expression of target genes was measured using 2X SYBR Green qPCR Master Mix (TaKaRa). The primers for qRT-PCR were used as follows:

MMP-9 Forward:  
5'-CCTGGAG ACCTGAGAACCAATCT-3'  
MMP-9 Reverse:  
5'-CCACCCGAGTGTA ACCATAGC-3'  
NF- $\kappa$ B Forward:  
5'-GGGAAGGAACGCTGTCAGAG-3'  
NF- $\kappa$ B Reverse:  
5'-TAGCCTCAGGGTACTCCATCA-3'

GAPDH Forward:  
5'-CAGTGCCAGCCTCGTCTCAT-3'  
GAPDH Reverse:  
5'-AGGGGCCATCCACAGTCTTC-3'

### Immunoblot analysis

The protein samples from each group of cells were collected and uploaded to 10% SDS-PAGE at a protein sample volume of 100  $\mu$ g per line. The proteins were subsequently transferred to PVDF membranes and closed with PBS containing 5% skim milk for 2 h and then overnight at 4 °C. The PVDF membranes were incubated with the primary antibody and then incubated with the secondary antibody for 1 h at room temperature. Finally, the bands were visualized using chemiluminescence and the Western blot bands were quantified using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). MMP-9,  $\beta$ -actin and secondary antibodies were purchased from Santa Cruz Biotechnology (Delaware Ave Santa Cruz, CA, USA).

### MTT and WST-1 assay for cell proliferation and activity

MCF-7 and MDA-MB-231 cells were inoculated into 96-well plates at a density of 5000 cells/well. According to standard experimental procedures, the cells in each group were transfected with liposome 3000 as a transfection reagent. The cell proliferation was detected using a cell counting kit (CCK8; 7Sea Biotech, Shanghai, China). The OD at 490 nm was measured at 24h, 48h, 72h and 96h after transfection using a FLUOstar OPTIMA microplate (BMG Labtech GmbH, Ortenberg, Germany) in order to measure cell proliferation. The cell activity was detected using the WST 1 assay (Roche Diagnostics) (18,19).

### Apoptosis assay

The cells of each group were resuspended in 100  $\mu$ L Annexin binding buffer and incubated with 5  $\mu$ L propidium iodide and 5  $\mu$ L FITC-Annexin V (BD, Franklin, NJ, USA). After incubation in the dark for 20 min at room temperature, Annexin binding buffer 400  $\mu$ L was added and the fluorescence intensity of propidium iodide and Annexin V was detected using flow cytometry (BeamCyte) and analyzed by CytoSYS 1.0 software.

## HE staining

The tumor tissue was fixed in 4% paraformaldehyde. The samples were embedded in paraffin, cut into 5- $\mu$ m-thick sections and stained with hematoxylin-eosin (HE) for histological and collagen analysis according to the experimental protocol (20).

## Data processing and graphing

All values were expressed as mean $\pm$ SEM. Data were statistically analyzed using the Mann-Whitney U test was used to compare two groups, the Kruskal-Wallis test followed by Dunn's multiple comparisons test using GraphPad Prism Software. The significance was set at  $p < 0.05$ .

## Results and discussion

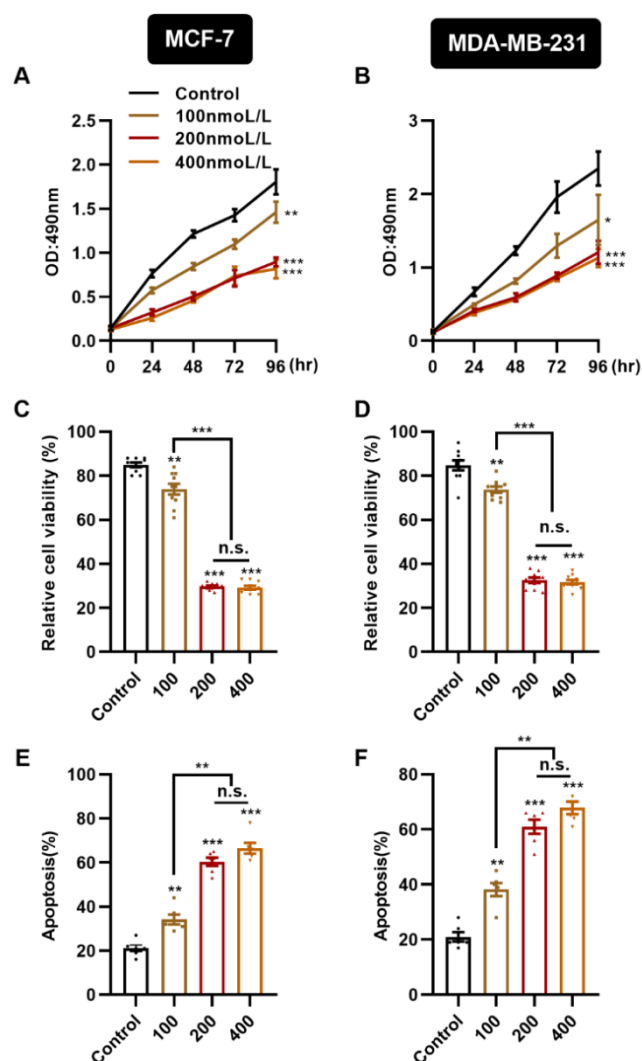
### SFN inhibited the proliferation and activity of MCF-7 and MDA-MB-231 in breast cancer cells

To investigate the molecular mechanism of SFN treatment for breast cancer, we first performed experiments using extracted SFN with MCF-7 and MDA-MB-231 breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with different concentrations (100, 200, 400 nmol/L) of extracted SFN. It was found that SFN at a dose of 200 nmol/L was the best inhibitor for MCF-7 proliferation, and the optimal inhibitor amount of SFN in MDA-MB-231 was the same as MCF-7 (Figures 1 A and B,  $P < 0.05$ ). SFN inhibited the activity of MCF-7 and MDA-MB-231 cells at the same dose as proliferation (Figures 1 C and D,  $P < 0.05$ ). Furthermore, SFN had the pro-apoptotic ability on both MCF-7 and MDA-MB-231 cells, and SFN had the strongest pro-apoptotic ability on MCF-7 and MDA-MB-231 cells at 400 nmol/L (Figures 1 E and F,  $P < 0.05$ ). The results of this experiment illustrate the inhibitory effect of SFN on breast cancer cells cultured in vitro, which predicts a therapeutic effect of SFN on breast cancer.

### SFN inhibited the expression and phosphorylation of MMP-9

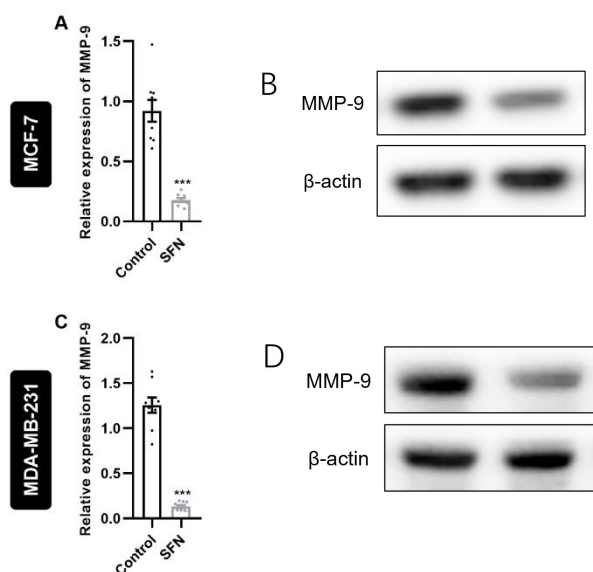
To verify the molecular mechanism of SFN inhibition in breast cancer cells, we examined the gene expression and protein phosphorylation of MMP-9 in MCF-7 and MDA-MB-231 cells at a dose of 200 nmol/L of SFN using RT-PCR and western blot. As shown in Figure 2A, there was a significant difference in the gene expression of MMP-9 between

the control and SFN-treated groups in MCF-7 cells, and the gene expression of MMP-9 was significantly lower in the SFN group ( $P < 0.05$ ). Also, western blot results showed differences in protein phosphorylation levels of MMP-9 between the control and SFN-treated groups (Figure 2B). The same trend was observed in MDA-MB-231 cells (Figures 2C and D,  $P < 0.05$ ). The results suggest a molecular mechanism for SFN inhibition of breast cancer cells in vitro, possibly through regulation of MMP-9 expression and phosphorylation.



**Figure 1.** Effect of different doses of SFN on proliferation, activity and apoptosis of MCF-7 and MDA-MB-231. (A and B) Proliferation inhibition of MCF-7 (A) and MDA-MB-231 (B) cells by different doses of SFN; (C and D) Activity inhibition of MCF-7 (C) and MDA-MB-231 (D) cells by different doses of SFN; (E and F) Promotion of apoptosis of MCF-7 (E) and MDA-MB-231 (F) cells by different doses of SFN. Kruskal-Wallis test followed by Dunn's multiple comparisons test. Error bars indicate SEM.



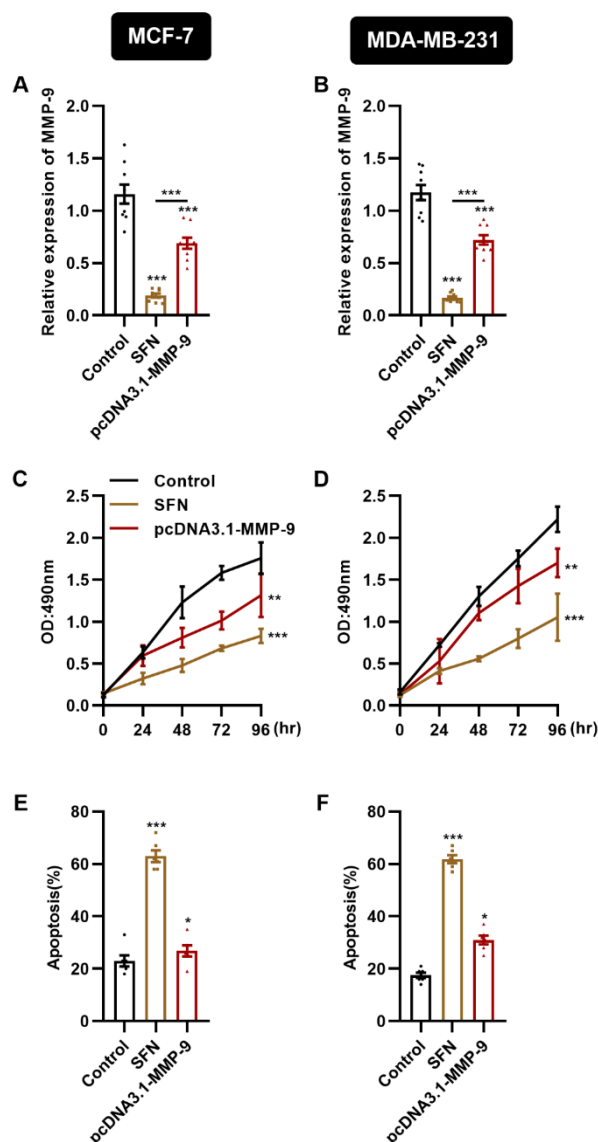


**Figure 2.** SFN decreased the expression level and phosphorylation level of MMP-9 in MCF-7 and MDA-MB-231 cells. (A and B) SFN decreased the expression level (A) and phosphorylation level (B) of MMP-9 in MCF-7 cells; (C and D) SFN decreased the expression level (C) and phosphorylation level (D) of MMP-9 in MDA-MB-231 cells. Mann-Whitney U test. Error bars indicate SEM.

### Activation of MMP-9 expression reduced the inhibitory effect of SFN on MCF-7 and MDA-MB-231 cells

To further demonstrate that SFN inhibited the proliferation of breast cancer cells by regulating MMP-9, we treated breast cancer cells with pcDNA3.1-MMP-9 overexpression plasmid. The cells were transfected with a pcDNA3.1-MMP-9 plasmid for 24 hours and then treated with 200 nmol/L SFN. As shown in Figure 3A, the inhibition of MMP-9 gene expression in MCF-7 was significantly diminished in the SFN-treated group at the dose of 200 nmol/L. In contrast, the addition of pcDNA3.1-MMP-9 plasmid significantly increased the MMP-9 expression inhibited by SFN, but it was still different from the control group. The same result was corroborated in MDA-MB-231 (Figure 3B,  $P < 0.05$ ). Meanwhile, MTT results showed that transfection of pcDNA3.1-MMP-9 plasmid significantly increased the proliferation ability of MCF-7 and MDA-MB-231 cells in the SFN group at 200 nmol/L dose (Figures 3C and D,  $P < 0.05$ ) and inhibited the apoptosis level of MCF-7 and MDA-MB-231 cells (Figures 3E and F,  $P < 0.05$ ). The results illustrate the possibility that SFN regulates breast cancer through MMP-9, while

activation of MMP-9 expression attenuates the inhibitory effect of SFN on breast cancer and that SFN inhibits MMP-9 expression.

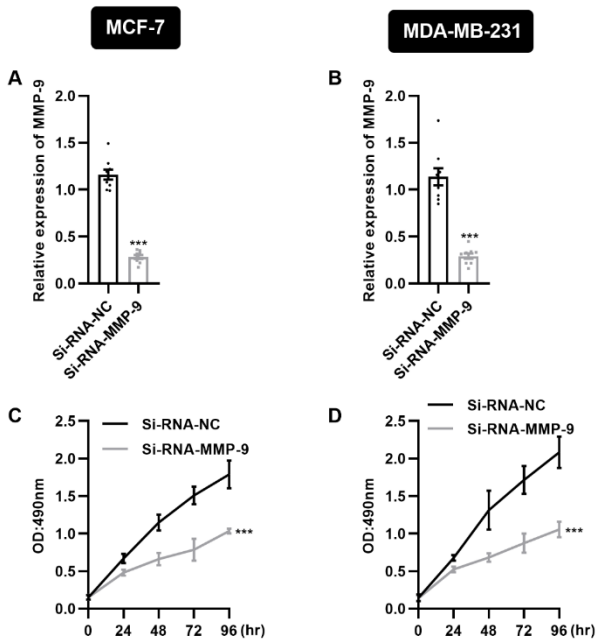


**Figure 3.** pcDNA3.1-MMP-9 restored the inhibitory effect of SFN on MCF-7 and MDA-MB-231 cells. (A and B) pcDNA3.1-MMP-9 restored the suppression of MMP-1 expression by SFN in MCF-7 (A) and MDA-MB-231 (B) cells. (C and D) pcDNA3.1-MMP-9 somewhat redeemed the inhibition of proliferation of MCF-7 (C) and MDA-MB-231 (D) cells by SFN. (E and F) pcDNA3.1-MMP-9 redeemed the pro-apoptotic effect of SFN on MCF-7 (E) and MDA-MB-231 (F) cells. Kruskal-Wallis test followed by Dunn's multiple comparisons test. Error bars indicate SEM.

### Si-RNA-MMP-9 inhibited the proliferation of MCF-7 and MDA-MB-231 cells

To verify the important role of MMP-9 in regulating the development of breast cancer, we again transfected MCF-7 and MDA-MB-231 cells with Si-

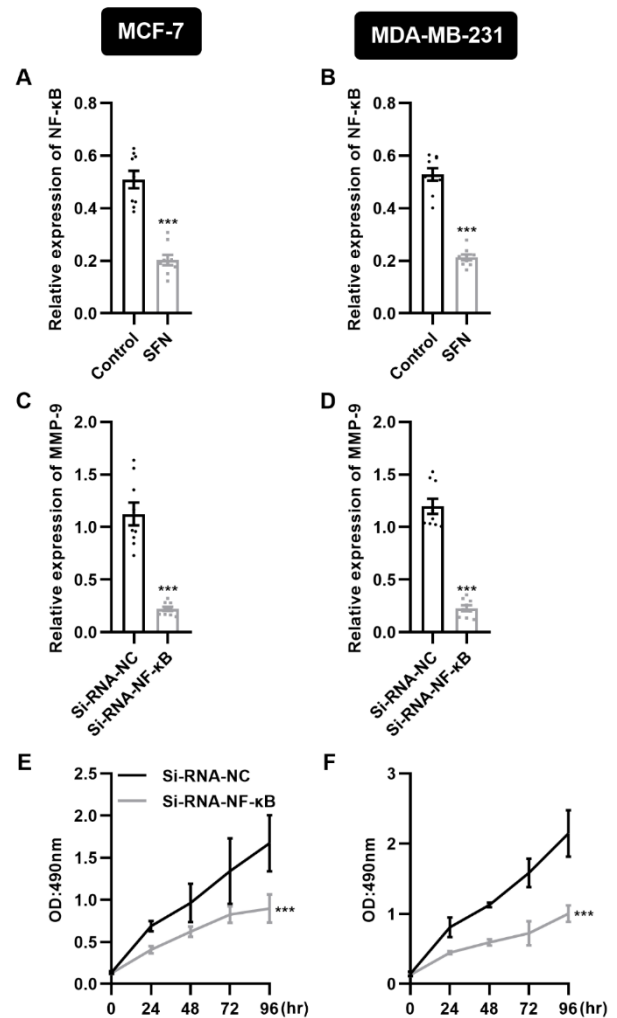
RNA-MMP-9. As shown in Figures 4A-B, the expression of MMP-9 was down-regulated in MCF-7 and MDA-MB-231 after transfection with Si-RNA-MMP-9. The results of the MTT analysis are shown in Figures 4C-D. Compared with MCF-7 and MDA-MB-231 cells transfected with Si-RNA-NC plasmid, the proliferation of breast cancer cells transfected with Si-RNA-MMP-9 was significantly attenuated ( $P < 0.05$ ). The results of this experiment illustrate the importance of the MMP-9 gene in regulating the development of breast cancer.



**Figure 4.** Si-RNA-MMP-9 plasmid inhibited the proliferation ability of MCF-7 and MDA-MB-231 cells. (A and B) Si-RNA-MMP-9 plasmid inhibited MMP-9 expression in MCF-7 (A) and MDA-MB-231 (B) cells; (C and D) Si-RNA-MMP-9 plasmid inhibited cell proliferation in MCF-7 (C) and MDA-MB-231 (D) cells. Mann-Whitney U test. Error bars indicate SEM.

In addition, there is growing evidence that the expression and activation of several MMPs (including MMP-9) are regulated by NF- $\kappa$ B activation in many human cancers. It has been found that SFN can inhibit NF- $\kappa$ B expression. Thus, SFN may also inhibit MMP-9 expression by suppressing the transcriptional activity of NF- $\kappa$ B protein and thus MMP-9 expression. PCR results showed that NF- $\kappa$ B mRNA levels were significantly reduced in MCF-7 and MDA-MB-231 cells treated with SFN (Figures 5A and B,  $P < 0.05$ ). To further confirm whether SFN regulated MMP-9 expression through the NF- $\kappa$ B

signaling pathway in human breast cancer cells, we transfected Si-RNA-NF- $\kappa$ B into MCF-7 and MDA-MB-231 cells. Our results showed that the expression of up-regulated MMP-9 in MCF-7 and MDA-MB-231 cells was inhibited using Si-RNA-NF- $\kappa$ B (Figures 5C and D,  $P < 0.05$ ). These results suggest that SFN may regulate MMP-9 expression through NF- $\kappa$ B. We also verified the mechanism of cell proliferation. The proliferation capacity of cells was significantly reduced after transfection of Si-RNA-NF- $\kappa$ B plasmids in MCF-7 and MDA-MB-231 cells (Figures 5E and F,  $P < 0.05$ ). These data suggest that SFN may regulate breast cancer development through the NF- $\kappa$ B/ MMP-9 signaling pathway.

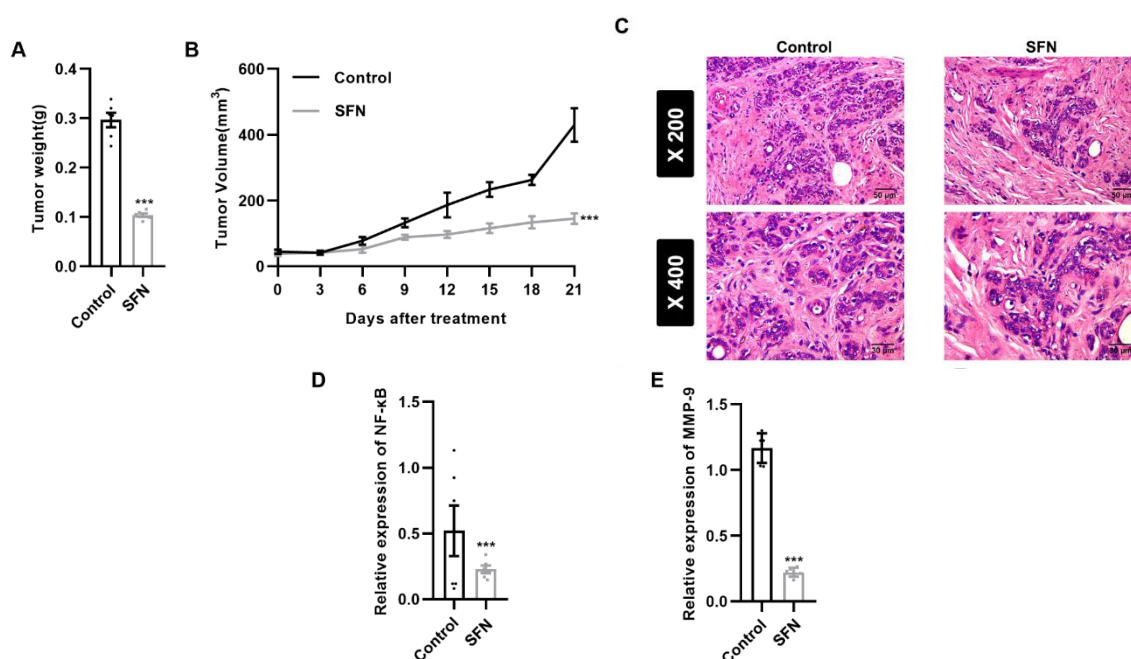


**Figure 5.** Inhibition of NF- $\kappa$ B expression suppressed MMP-9 expression. (A and B) Si-RNA-NF- $\kappa$ B inhibited NF- $\kappa$ B expression in MCF-7 (A) and MDA-MB-231 (B) cells; (C and D) Si-RNA-NF- $\kappa$ B inhibited MMP-9 expression in MCF-7 (C) and MDA-MB-231 (D) cells; (E and F) Si-RNA-NF- $\kappa$ B inhibited the proliferation ability of MCF-7 (E) and MDA-MB-231 (F) cells. Mann-Whitney U test. Error bars indicate SEM.

## SFN improved disease signs in rats with breast cancer

To further confirm the anti-breast cancer effect of SFN, we utilized a xenograft mouse model of MDA-MB-231 cells. MDA-MB-231 cells ( $1 \times 10^7$  cells per mouse) were injected subcutaneously into the right dorsum of nude mice. When the tumor volume reached approximately  $50 \text{ mm}^2$ , the mice were randomly divided into two groups. One group received saline control treatment (2% DMSO / 8% olive oil in saline) and the other group received 50 mg/kg SFN treatment for 21 consecutive days. Subsequently, the mice were humanely executed, and

the tumors were collected and weighed. As shown in Figures 6A and B, the treatment with SFN significantly inhibited the tumor growth ( $P < 0.05$ ). In addition, hematoxylin and eosin (H&E) staining showed that SFN significantly reduced the density of tumor cells (Figure 6C). RT-PCR assay revealed that NF- $\kappa$ B and MMP-9 expression was reduced in SFN-treated rats compared with the saline control treatment group (Figures 6D-E,  $P < 0.05$ ). The results illustrate that SFN treatment has anti-mammary cancer effects in mice and is achieved by inhibiting NF- $\kappa$ B and MMP-9 expression.



**Figure 6.** SFN improved tumor histopathology in xenograft tumor model mice. (A and B) SFN reduced tumor size and volume in xenograft tumor model mice; (C) SFN improved tumor histopathology in xenograft tumor model mice; (D and E) SFN decreased NF- $\kappa$ B (D) and MMP-9 (E) gene expression in xenograft tumor model mice. Mann-Whitney U test. Error bars indicate SEM.

## Conclusions

SFN is one of the main active compounds of plant origin with a wide range of pharmacological activities (10). SFN was found to be converted from cauliflower shoots and mainly through thioglucoside (7). The chemopreventive properties of SFN against cancer are achieved by blocking and inhibiting effects (21). The blocking function of SFN is achieved by inhibiting the conversion of carcinogens into phase 1 metabolizing enzymes and by inhibiting phase 2 metabolic enzymes that promote carcinogenesis (22). Some studies have identified the inhibitory role of SFN in regulating the

activity of a variety of cells to inhibit the growth of transformed cells (22). In terms of gene regulation, SFN can inhibit the binding activity of NF- $\kappa$ B expressed through the stimulation of voltarol (12-O-tetradecanoyl phorbol-13-acetate) (23). SFN is also able to inhibit smooth muscle cell proliferation and migration by decreasing the activity of MMP-9 (24).

In this study, we demonstrated that SFN could inhibit the proliferation and activity of breast cancer cells and improve tumor histopathology in xenograft tumor model mice by regulating the expression of NF- $\kappa$ B/ MMP-9. NF- $\kappa$ B is a molecule that plays a

dominant role in inflammatory cytokine induction (25). It is also involved in the regulation of immune function, cell cycle control and apoptosis (25). In contrast, MMP-9 is highly expressed at sites of inflammation and is involved in the pathogenesis of several chronic inflammatory diseases (26). NF- $\kappa$ B is upstream of MMP-9 and involved in the regulation of MMP-9 expression. It has been found that the inhibitory activity of MMP-9 is positively correlated with NF- $\kappa$ B (27-29). In breast cancer cells MCF-7 and MDA-MB-231, the proliferation ability of the cells was inhibited by different doses of SFN treatment, and the strongest inhibition of cell proliferation was observed when using extracted SFN at a concentration of 200 nmoL/L. The assay of cell activity and apoptosis revealed that SFN could effectively inhibit the activity of breast cancer cells and promote apoptosis. To investigate the molecular mechanism of this phenomenon, we examined the expression and protein phosphorylation levels of MMP-9 using RT-PCR and western blot assays. The results showed that SFN treatment significantly inhibited the expression and protein phosphorylation of MMP-9. The transfection of pcDNA3.1-MMP-9 plasmid restored the inhibitory effect of SFN on breast cancer cells, while the transfection of Si-RNA-MMP-9 had the same inhibitory effect as SFN on breast cancer cells. Therefore, we suggest that SFN can inhibit the development of breast cancer by suppressing the expression of MMP-9.

The activity of MMP-9 is associated with the infiltration process of tumor cells (30). However, the exact mechanism of MMP-9 activation in malignant tissues has not been determined (31). Many purified proteases, including trypsin, chymotrypsin, MMP-2, tissue kinase releasing enzyme, trypsin 2, fibrinolytic enzymes, MMP-7, MMP-13 and MMP-3 are able to activate MMP-9 in vitro (31). NF- $\kappa$ B is the upstream gene of MMP-9 and is able to regulate the expression of MMP-9 (15, 32-34). Therefore, we again verified whether the regulation of MMP-9 by SFN was achieved through the regulation of NF- $\kappa$ B. The results revealed that SFN was able to inhibit NF- $\kappa$ B expression. Also, transfection of Si-RNA-NF- $\kappa$ B inhibited the expression of MMP-9 in breast cancer cells. Therefore, we speculate that SFN can inhibit the development of breast cancer by regulating the expression of the NF- $\kappa$ B/ MMP-9 pathway.

Finally, to demonstrate the effectiveness of SFN in the treatment of breast cancer, we constructed a breast cancer cell xenograft tumor model using nude mice and breast cancer cells MDA-MB-231. Then, we treated model mice with 50 mg/kg of SFN. The results showed that SFN was effective in reducing tumor size and volume, and improved the pathological condition of tumor tissues as well as inhibited NF- $\kappa$ B/ MMP-9 expression in the model mice.

In conclusion, this study demonstrated the effectiveness of SFN in treating breast cancer. Moreover, SFN can inhibit the growth proliferation and activity of breast cancer cells by regulating the expression of the NF- $\kappa$ B/ MMP-9 signaling pathway to treat breast cancer.

### Acknowledgments

Not applicable.

### Interest conflict

The authors declare that they have no conflict of interest.

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