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Sulforaphane epigenetically demethylates the CpG sites of the miR-9-3 promoter and reactivates miR-9-3 expression in human lung cancer A549 cells

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Abstract

Increasing evidence suggests that epigenetic aberrations contribute to the development and progression of cancers such as lung cancer. The promoter region of miR-9-3 was recently found to be hypermethylated in lung cancer, resulting in down-regulation of miR-9-3 and poor patient prognosis. Sulforaphane (SFN), a natural compound that is obtained from cruciferous vegetables, has potent anticancer activities. In this study, we aimed to investigate the effect of SFN on restoring the miR-9-3 level in lung cancer A549 cells through epigenetic regulation. DNA methylation of the miR-9-3 promoter was examined using bisulfite genomic sequencing and methylated DNA immunoprecipitation analysis. The expression levels of miR-9-3 and several epigenetic modifying enzymes were measured using quantitative real-time polymerase chain reaction and Western blotting, respectively. The transcriptional activity of the miR-9-3 promoter was evaluated by patch methylation, and histone modifications were analyzed using chromatin immunoprecipitation (ChIP) assays. We found that CpG methylation was reduced in the miR-9-3 promoter and that miR-9-3 expression was increased after 5 days of treatment with SFN. *In vitro* methylation analysis showed that the methylated recombinant construct exhibited lower luciferase reporter activity than the unmethylated counterpart. ChIP assays revealed that SFN treatment increased H3K4me1 enrichment at the miR-9-3 promoter. Furthermore, SFN treatment attenuated enzymatic DNMT activity and DNMT3a, HDAC1, HDAC3, HDAC6 and CDH1 protein expression. Taken together, these findings indicate that SFN may exert its chemopreventive effects partly through epigenetic demethylation and restoration of miR-9-3.

Keywords

Sulforaphane; miR-9-3; Lung cancer; Epigenetics; DNA methylation

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Conflict of interest statement

No potential conflicts of interest are disclosed.

1. Introduction

Lung cancer is the most common cancer worldwide, with an estimated 526,510 cases in the United States and 224,390 cases in other countries diagnosed in 2016 [1]. Environmental pollution and genetic mutations are the most well-known risk factors for the development of lung cancer [2–4]. In addition to these risk factors, epigenetic changes are involved in lung tumorigenesis based on accumulating evidence [5–7]. Unlike genetic mutation of the DNA sequence, epigenetic dysregulation involves reversible alterations to the chromatin structure that do not change the DNA sequence. The reversible nature of epigenetic aberrations has motivated efforts to develop predictive biomarkers and chemotherapeutic targets for lung cancer [5,8–10].

Epigenetic mechanisms are classified into three types: DNA methylation, histone modifications and noncoding RNA modulations [5,11]. DNA methylation that occurs in CpG islands not only is related to tumor progression but also is a candidate biomarker for the early detection of lung cancer [12,13]. Several tumor suppressor genes, such as deleted in lung and esophageal cancer 1 (*DLEC1*), integrin subunit alpha 9 (*ITGA9*) and mutL homolog 1 (*MLH1*), are silenced by hypermethylation in lung cancer [14]. Histone modifications, which include methylation, acetylation, phosphorylation and ubiquitination, constitute another type of epigenetic mark [11]. Of these modification types, methylation and acetylation have been extensively investigated [15]. Histone acetylation or methylation of lysine 4 on H3 (H3K4) promotes gene activation, whereas deacetylation or methylation of H3K27 promotes gene silencing [11,16]. Better understanding of epigenetic aberrations offers effective targets for lung cancer therapy by reactivating epigenetically silenced genes. Moreover, DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors may be useful as promising antitumor agents in the treatment of lung cancer.

Sulforaphane (SFN), a natural compound that is obtained from cruciferous vegetables and that is known for its potent anticancer activity, has been identified as an HDAC inhibitor [17,18]. In regards to its mechanism of action, we previously demonstrated that SFN enhances Nrf2 expression by decreasing CpG methylation of the *Nrf2* promoter in prostate cancer TRAMP C1 cells and in mouse epidermal JB6 cells [19,20], suggesting that SFN may exert a chemopreventive effect *via* epigenetic regulation.

MicroRNAs (miRNAs), small noncoding RNAs that are approximately 22 nucleotides long, function as posttranscriptional regulators of gene expression by binding to the 3′-untranslated regions (UTR) [21]. miR-9 includes three family members: miR-9-1, miR-9-2 and miR-9-3, which are encoded by chromosome 1q22, chromosome 5q14.3 and chromosome 15q26.1, respectively [22]. Recently, miR-9-3 has been identified to be hypermethylated in its promoter region, resulting in its down-regulation in human cancer [22–26]. Importantly, lung cancer patients with methylated miR-9-3 have shorter disease-free survival, progression-free survival and overall survival rates [23,27]. To date, the effect of SFN on lung cancer *via* the modulation of miRNA promoter methylation remains unexplored. In this study, we aimed to investigate whether SFN could restore miR-9-3 expression in human lung cancer A549 cells by affecting epigenetic modifications.

2. Materials and methods

2.1. Materials

SFN, 5-Aza-2′-deoxycytidine (5AZA), trichostatin A (TSA), ampicillin, bovine serum albumin (BSA) and a protein inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and trypsin–EDTA (0.25%) were purchased from Gibco (Carlsbad, CA, USA). The anti-β-actin primary antibody and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against DNMT1, DNMT3a, DNMT3b and HDACs 1–8 were purchased from Cell Signaling Technology (Boston, MA, USA). Antibodies against PR/SET domain 1 (PRDM1), forkhead box O1A (FOXO1A) and cadherin-1 (CDH1) were purchased from Abcam (Cambridge, MA, USA).

2.2. Cell culture and treatment

Human lung adenocarcinoma (A549), human embryonic kidney (HEK293) and colorectal adenocarcinoma (HT29) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were routinely cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. A549 cells were seeded in 10-cm dishes at a density of 1×10^5 cells/dish for 24 h and subsequently treated with either 0.1% DMSO, different concentrations of SFN or a combination of 5AZA (2.5 μM) and TSA (100 nM) for 5 days.

2.3. Cell viability assay

A549 cells were seeded in 96-well plates (1000 cells/well). After 24 h, the cells were treated with various concentrations of SFN ranging from 0.625 μM to 40 μM. The CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay) was used to assess cell viability (Promega, Madison, WI, USA) on days 1, 3 and 5.

2.4. Bisulfite genome sequencing (BGS)

For the DNA methylation analysis, we used BGS, which is described in detail in our previous report [28]. Briefly, 500 ng of genomic DNA was bisulfite converted using the EZ DNA Methylation-Gold Kit following the manufacturer's instructions (Zymo Research, Orange, CA, USA). The bisulfite-modified DNA was amplified using primers, as described previously, to amplify the fragment between −129 and +232 bp of the promoter region of miR-9-3 [23]. The polymerase chain reaction (PCR) products were purified and cloned into a pCR4 TOPO vector using the TOPO TA Cloning Kit (Thermo Fisher Scientific, Rockford, IL, USA). Plasmid DNA from 10 randomly selected clones was isolated and sequenced (Genewiz, Piscataway, NJ, USA).

2.5. Methylated DNA immunoprecipitation (MeDIP) analysis

To confirm the BGS results, MeDIP was carried out using the Methylamp Methylated DNA Capture Kit (EpiGentek, Farmingdale, NY, USA) as described in our previous work [19,28,29]. Briefly, the DNA that was extracted from the treated cells was sonicated using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ, USA) to shear the DNA into 200-to 1000-

bp fragments. The sonicated DNA was incubated at 95°C for 2 min and immunoprecipitated with anti-5-methylcytosine at room temperature for 2 h. After the DNA was washed and purified, the methylation status was quantified by quantitative PCR (qPCR).

2.6. RNA extraction and quantitative real-time PCR

Total RNA was extracted from A549 cells using the GeneJET RNA Purification Kit (Thermo Fisher Scientific). cDNA was synthesized from 1.5 μg of total RNA using the miScript II RT Kit (Qiagen, Valencia, CA, USA). Quantitative real-time PCR was performed using the miScript SYBR Green PCR Kit and miScript Primer Assays according to the manufacturer's instructions (Qiagen). The relative expression of precursor miR-9-3 was analyzed using the comparative Ct method [30]. RNU6 was used as an internal control.

2.7. Western blotting

After washing with phosphate-buffered saline (PBS), the treated cells were harvested with trypsin and lysed using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Boston, MA, USA) containing a protease inhibitor cocktail. Protein concentrations were measured using the bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, USA), and 20 μg of the total protein was resolved by 4%–15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA). Following electrophoresis, the proteins were transferred to a TransBolt Turbo Midi-size polyvinylidene difluoride membrane (Bio-Rad). The membrane was then blocked with 5% BSA and subsequently incubated with specific primary antibodies and horseradish-peroxidaseconjugated secondary antibodies in a PBS–0.1% Tween 20 buffer. The bands were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and analyzed using ImageJ version 1.48d.

2.8. Plasmid construction and luciferase reporter assay

Genomic DNA was isolated from lung cancer A549 cells, and the segment of the miR-9-3 promoter (−129 ~ +232 bp) was amplified using the following primers: 5′-AATGCATGTGTGCGTGTGTCTGTCCATCCC-3′ (forward) and 5′-CCACTAGTGGCACTGCAAGTGTCCCCAGAGA-3′ (reverse). The PCR products were digested with *Nsi* I and *Spe* I and then inserted into the pCpGfree-promoter-Lucia vector (InvivoGen, San Diego, CA, USA). After sequencing confirmation, the recombinant plasmid was treated with methyltransferase M.Sss I at 37°C for 2 h (New England Biolabs, Beverly, MA, USA). The methylated plasmid was purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The efficiency of the methylation process was verified using the methylation-insensitive *Hpa* II and *Hha* I restriction endonucleases.

A549, HEK293 and HT29 cells were seeded in 12-well plates for 24 h, and 500 ng of the recombinant plasmids was transfected using Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. A total of 50 ng of pGL 4.13 was co-transfected as the internal control. After 24 h, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a MAGnify Chromatin Immunoprecipitation system according to manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, chromatin from cells was fixed with 1% formaldehyde and sonicated to generate 200- to 500-bp fragments using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ, USA). The sonicated solutions were incubated with protein A/G Dynabeads and specific antibodies against H3K4me1 (ab8895, Abcam, Cambridge, MA, USA) or rabbit IgG overnight at 4°C. After undergoing reverse cross-linking, the DNA was purified using the ChIP DNA Clean Concentrator (Zymo Research) according to the manufacturer's instructions. Enrichment of the diluted DNA was quantified by qPCR using the following primers : 5′-AGATCGCCACGTCCTATCAC-3′ (forward) and 5′-ACCCAAACTCCTCCGTCTTC-3′ (reverse).

2.10. HDAC and DNMT activity assays

HDAC activity was evaluated using the Epigenase HDAC Activity/Inhibition Direct Assay Kit, and DNMT activity was evaluated using the EpiQuik DNMT Activity/Inhibition Assay Ultra Kit (EpiGentek). The nuclear protein was isolated using the NEPER Nuclear and Cytoplasmic Protein Extraction Kit from Thermo Fisher Scientific. The method for calculating relative HDAC or DNMT activity was described previously [20].

2.11. Statistical analysis

All analyses were performed using SPSS software, version 19.0 (Chicago, IL, USA). Data are presented as the mean±standard error of the mean (S.E.M.). The statistical analyses were carried out using one-way analysis of variance (ANOVA) or Student's t test. P values less than .05 were considered statistically significant.

3. Results

3.1. Cytotoxicity of SFN in A549 cells

The cytotoxicity of SFN against A549 cells was measured using MTS assay. Cell viability was reduced in a time- and dose-dependent manner after 1, 3 and 5 days of SFN treatment (Fig. 1). To avoid toxicity, we chose the concentrations with cell survival rates that exceeded 90% (i.e., 2.5 μM and 5.0 μM) for further experimentation.

3.2. SFN decreased CpG methylation in the promoter region of miR-9-3

A previous report revealed that the miR-9-3 promoter was highly methylated and subsequently down-regulated in lung cancer cell lines [23]. In this study, we performed BGS and MeDIP assays to examine whether SFN could reverse miR-9-3 promoter methylation. As shown in Fig. 2A, the BGS assay revealed a hypermethylated region in the miR-9-3 promoter (−129 to+232 bp) in A549 cells, with methylation at 62.1% of the CpG sites. After treatment with 2.5 μM SFN, 5.0 μM SFN or 2.5 μM 5AZA plus 100 nM TSA (positive control) for 5 days, the percentage of methylated CpG sites significantly decreased ($P<.01$, one-way ANOVA).

To verify the BGS results, MeDIP assay, which is a technique that utilizes an anti-5-methylcytosine antibody to capture methylated genomic DNA without bisulfite conversion, was performed. The enriched methylated fractions were then measured by qPCR. As shown in Fig. 2B, the methylated DNA ratio was significantly reduced in the 2.5 μM SFN, 5.0 μM SFN and 5AZA/TSA treatment groups compared with the control group ($P<.01$, one-way ANOVA). Taken together, these data indicate that SFN can decrease CpG methylation in the miR-9-3 promoter region in A549 cells.

3.3. SFN increased H3K4me1 enrichment at the miR-9-3 promoter

Evidence for epigenetic cross-talk between DNA methylation and histone modification is frequently observed in the regulation of gene expression [31–33]. In addition to DNA methylation, H3K4me1 enrichment at the miR-9-3 promoter in A549 cells was investigated *via* ChIP analysis. H3K4me1 enrichment was increased after SFN treatment compared with control treatment, as shown in Fig. 2C (P=.01, Student's *t* test).

3.4. *In vitro* methylation suppressed the transcriptional activity of the miR-9-3 promoter

Because promoter CpG methylation can suppress gene transcription, we constructed luciferase reporter plasmids that contained the miR-9-3 promoter region (−129 to +232 bp) to examine whether the CpG sites in this region could influence transcriptional activation. Compared with the unmethylated construct, the methylated construct showed significantly decreased luciferase reporter expression by 62%, 73% and 82% in the A549, HEK293 and HT29 cell lines, respectively (Fig. 3A). Moreover, we examined the level of miR-9-3 after treatment with SFN using qPCR. As shown in Fig. 3B, miR-9-3 expression was induced by 5.0 μM SFN treatment and 5AZA/TSA treatment ($P<.01$).

3.5. SFN diminished the expression and activity of epigenetic modifying enzymes

To explore the epigenetic mechanism by which SFN decreased miR-9-3 promoter methylation and increased miR-9-3 levels, we examined the expression and activity of epigenetic modifying enzymes, including DNMTs and HDACs. As shown in Fig. 4, SFN decreased the protein levels of DNMT3a but not of DNMT1 or DNMT3b. Additionally, treatment with SFN significantly reduced the protein levels of HDAC1, HDAC3 and HDAC6. However, no significant changes in HDAC2, HDAC4, HDAC5, HDAC7 or HDAC8 expression were observed following SFN treatment (Fig. 5). Interestingly, 2.5 μM SFN significantly decreased DNMT activity, whereas 5.0 μM SFN had no effect on DNMT activity (Fig. 6A). No significant inhibition of HDAC activity by SFN treatment was observed (Fig. 6B).

3.6. SFN decreased the protein expression of CDH1

Previous studies have demonstrated that miRNAs function as posttranscriptional regulators of gene expression by binding to its 3′ UTR [21]. In this study, we selected three target genes of miR-9 (i.e., *PRDM1*, *FOXO1* and *CDH1*) to examine whether SFN treatment can influence the protein levels. As shown in Fig. 7A and B, the protein expression of CDH1 was decreased after treatment with 2.5 μM and 5.0 μM SFN ($P<.01$). The protein levels of PRDM1 and FOXO1A, however, were not influenced following SFN treatment.

4. Discussion

miR-9-3 is differently expressed in human cancer. Some studies reported that miR-9 was up-regulated and promoted cancer metastasis [34,35], whereas some studies reported that miR-9 was down-regulated, with a function of suppressing proliferation and promoting apoptosis [36,37]. Although it is difficult to decipher the exact reason for the conflicting results, one possible reason may be that miR-9-3 has a dual role in tumorigenesis.

It is well known that numerous coding genes are silenced at the transcriptional level in lung cancer due to hypermethylation of the promoter region. Recent studies have revealed that noncoding RNAs, mainly miRNAs, can harbor CpG methylations, which result in the down expression of these RNAs [22–26,38]. miR-9-3, a kind of miRNA, is specifically methylated in a number of human cancers, including lung cancer [23,26,39–42]. The methylated CpG island is located between nucleotides −129 and +232 relative to the transcriptional start site of miR-9-3 [23]. Methylation of miR-9-3 increases the risk of metastatic recurrence and decreases progression-free survival, disease-free survival and overall survival among cancer patients [23,27,43]. Moreover, miR-9-3 methylation may be a biomarker for the detection of hepatocellular carcinoma [26], bladder cancer [40], and oral and oropharyngeal squamous cell carcinomas [42]. Demethylation of miR-9-3 can up-regulate its expression; can inhibit cell proliferation, migration and invasion; and can promote apoptosis through targeting the NFκB pathway [24,37,44]. Based on this background, we hypothesized that it might be useful for personalized therapies for cancer patients that incorporate epigenetic restoration of miR-9-3 expression. In this study, SFN was used to determine whether it can epigenetically demethylate the CpG sites of the miR-9-3 promoter and reactivate miR-9-3 expression in human lung cancer A549 cells.

In our study, BGS analysis in A549 cells revealed that 62.1% of CpG sites in the miR-9-3 promoter were methylated (Fig. 2A), and this finding is consistent with the results reported by Kitano and colleagues [27]. miR-9-3 promoter methylation was decreased by treatment with 2.5 μM or 5.0 μM SFN (Fig. 2A and B), and up-regulation of miR-9-3 was observed following SFN treatment (Fig. 3B). Further analysis showed that SFN-mediated demethylation is associated with the inhibition of both DNMT3a protein expression and DNMT enzymatic activity (Figs. 4 and 6A). The key roles of DNMTs in regulating DNA methylation have been demonstrated by several research groups. Clinically, all subtypes of DNMTs (DNMT1, DNMT3a and DNMT3b) are highly expressed in lung cancer, and the overexpression of DNMTs may lead to the hypermethylation of multiple tumor suppressor gene promoters, leading to tumorigenesis and poor prognosis [45,46]. Knockdown of DNMT1 can reduce methylation and can alleviate the decrease in miR-9 expression both in vivo and in vitro [26,47]. DNMT inhibitors can also induce radiosensitivity in A549 cells [48].

Regarding the effect of SFN on DNMTs, Ali Khan et al. reported that SFN inhibits DNMT activity and diminishes DNMT3b expression in HeLa cells [49]. Meeran et al. reported that SFN inhibits DNMT1 and DNMT3a expression in a dose-dependent manner in human breast cancer cells [50]. In the androgen-dependent prostate cancer LNCaP cell line, SFN decreases DNMT1 and DNMT3b expression [51,52]. In the androgen-independent prostate

cancer PC3 cell line, SFN decreases the expression of all subtypes of DNMTs [51]. Our previous studies revealed that SFN decreases DNMT1 and DNMT3a expression levels in prostate cancer TRAMP C1 cells and DNMT1, DNMT3a and DNMT3b levels in mouse epidermal JB6 cells [19,20]. The data from this study, together with previous evidence, suggest that the SFN-mediated inhibition of DNMT expression may partly play a role in facilitating the demethylation of the miR-9-3 promoter, although the exact mechanism requires further investigation.

In addition to altering DNA methylation, SFN may alter histone modifications because SFN functions as an HDAC inhibitor [17,18]. Rajendran et al. reported a significant reduction in HDAC1, HDAC2, HDAC3 and HDAC8 protein expression in SFN-treated human colon cancer cells [53]. Clarke et al. reported that SFN stimulates apoptosis and cell cycle arrest by inhibiting HDAC activity and class I and class II HDACs and by increasing H3 acetylation at the *P21* promoter in prostate cancer cells [54]. Comparable results have also been observed in vivo [55]. In addition to histone acetylation, SFN plays crucial roles in the regulation of histone methylation. For example, SFN treatment increases the levels of active chromatin marks, namely, acetyl-H3, acetyl-H3K9 and acetyl-H4, and decreases the levels of repressive chromatin marks, namely, trimethyl-H3K9 (H3K9me3) and trimethyl-H3K27 (H3K27me3), in breast cancer and prostate cancer cells [19,50]. Similarly, SFN reduces H3K27me3 levels in skin cancer cells [56]. Moreover, lung cancer patients with high H3K27me3 expression have longer overall survival and a better prognosis [57]. Based on these data, we hypothesized that histone modifications may contribute to SFN-induced activation of miR-9-3, and our results confirmed this hypothesis. SFN treatment in A549 cells increased H3K4me1 enrichment at the miR-9-3 promoter and decreased HDAC1, HDAC3 and HDAC6 expression (Figs. 2C and 5).

Previous work has shown that miR-9 is involved in carcinogenesis via binding to the 3′ UTR of several target genes [34,47,58–61]. For example, Nie et al. reported that miR-9 may contribute to the pathogenesis and phenotype maintenance of Hodgkin/Reed– Sternberg cells *via* down-regulation of *PRDM1* [60]. Liu et al. reported that miR-9 can promote cell proliferation, migration and invasion *via* down-regulating *FOXO1* or *CDH1* [59,61]. In this study, we found that SFN decreased the protein levels of CDH1 (Fig. 7A and B), confirming the epigenetic effects of SFN on miR-9-3 being translated to downstream signaling pathway. Taken together, our findings indicate that SFN may epigenetically restore miR-9-3 expression by inhibiting the protein levels of DNMTs, HDACs and its target gene *CDH1*. A similar mechanism was also reported by Martin et al. who found that SFN inhibited cell viability and induced apoptosis of colon cancer cells through epigenetic modulation of miR-21 and human telomerase reverse transcriptase [62].

In conclusion, this study demonstrates that SFN can restore miR-9-3 levels by altering CpG methylation and that H3K4me1 modification is enriched at the miR-9-3 promoter in A549 cells. These findings provide a novel mechanism through which SFN can act as an epigenetic modulator to prevent lung cancer.

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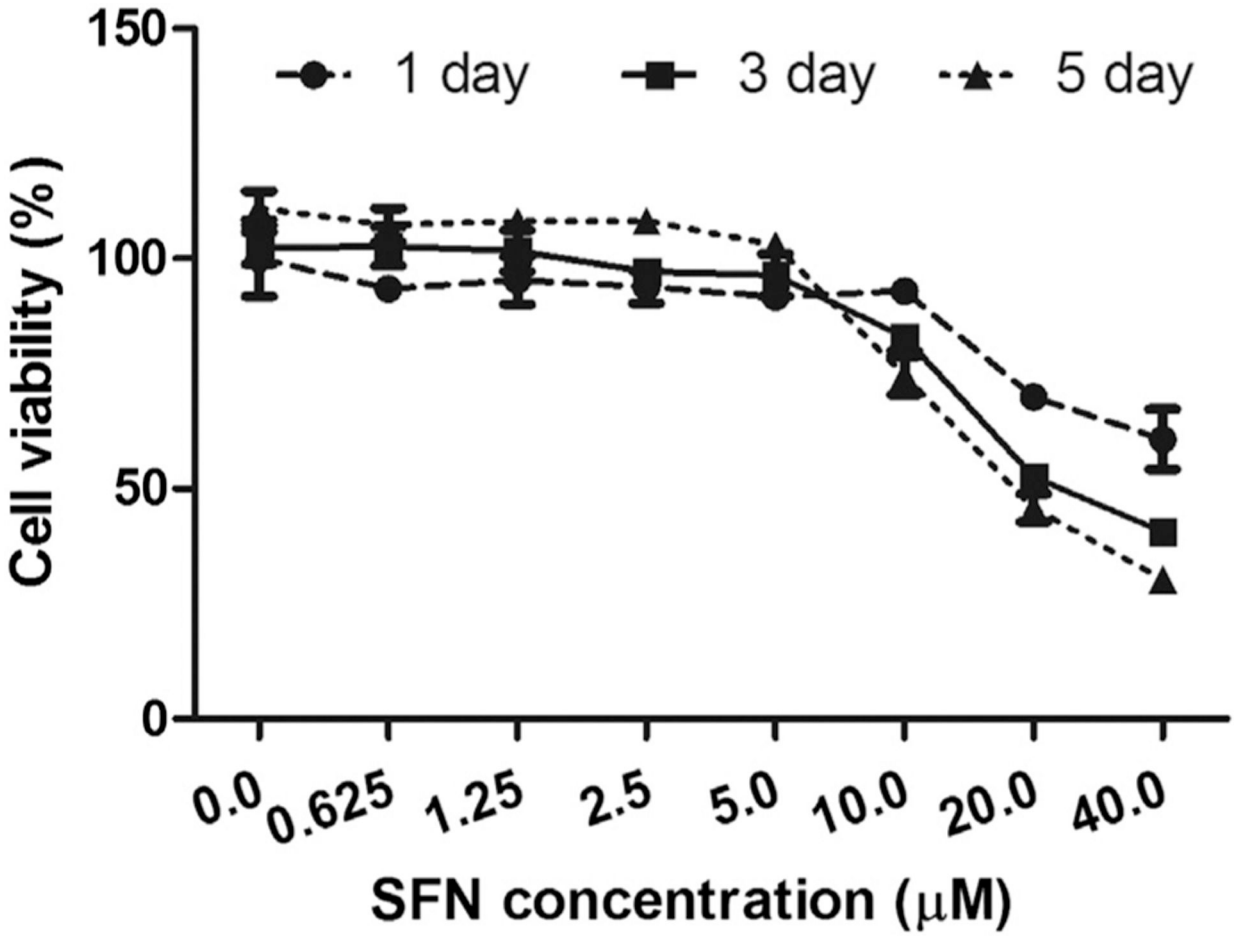

Fig. 1.

Cytotoxicity of SFN against A549 cells. Cells were treated with various concentrations of SFN for 1, 3 and 5 days. The MTS assay was performed to assess cell viability. Data are presented as the mean ± S.E.M.

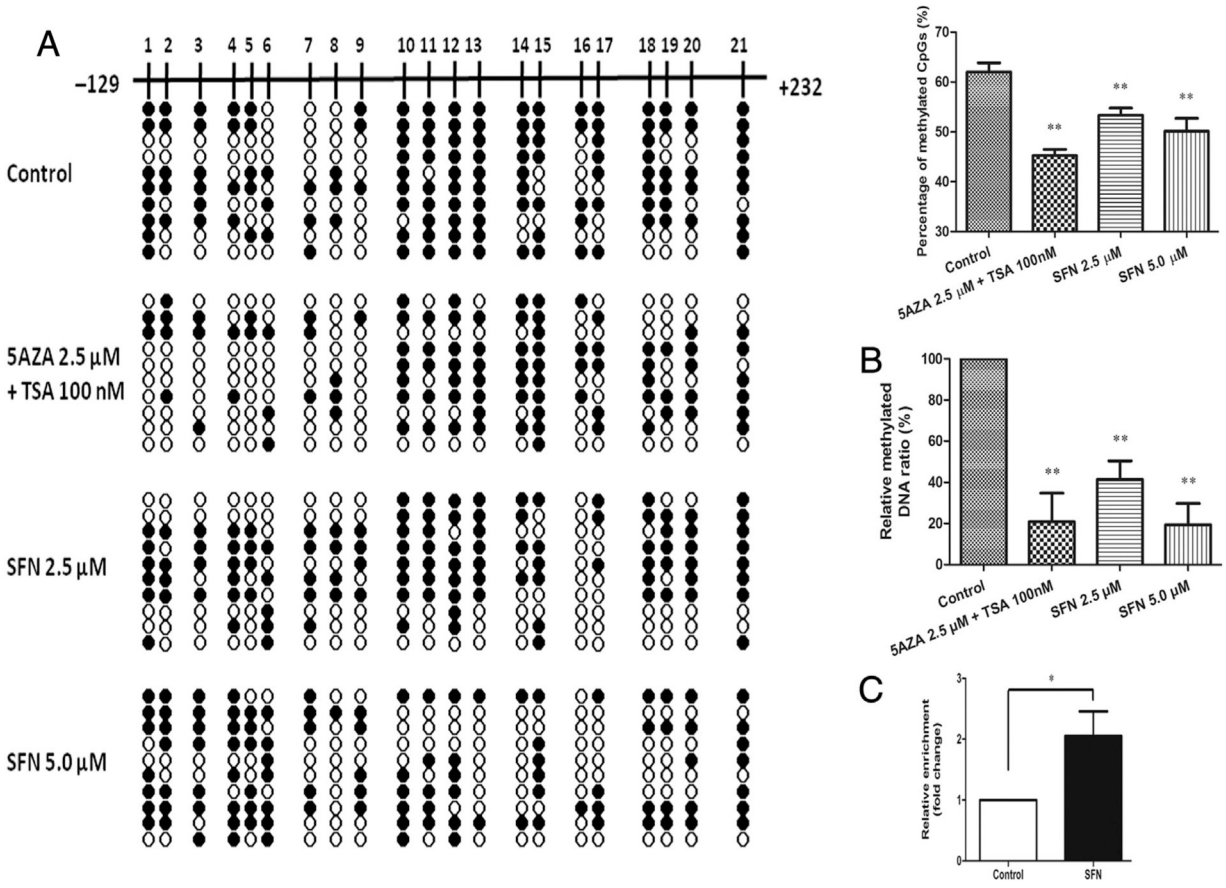

Fig. 2.

SFN alters DNA methylation and histone modification of the miR-9-3 promoter. A549 cells were treated with various concentrations of SFN or 5AZA/TSA for 5 days. The analyzed CpG sites are located within the −129-bp to +232-bp region of the miR-9-3 promoter. (A) BGS was used for DNA methylation analysis. Black spots denote methylated CpGs, and hollow dots denote unmethylated CpGs. (B) The MeDIP assay was used to estimate the enrichment of the methylated DNA fragments captured by the anti-5-methylcytosine antibody. (C) The ChIP assay was used to assess H3K4me1 enrichment at the miR-9-3 promoter. **P*<.05 and ***P*<.01 compared with the control.

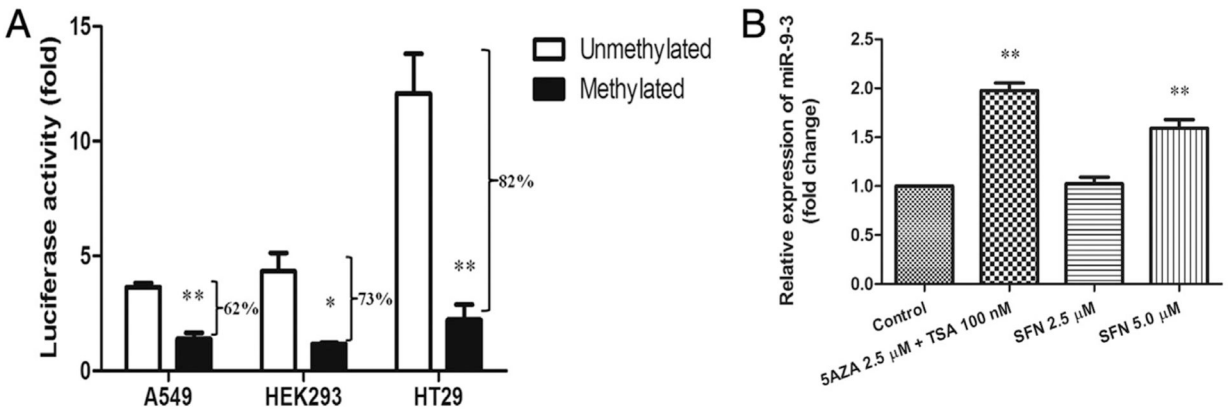

Fig. 3.

SFN increases miR-9-3 expression. (A) *In vitro* methylation suppressed the transcriptional activity of the miR-9-3 promoter. The miR-9-3 promoter region (−129 to +232 bp) was cloned into the pCpGfree-promoter-Lucia vector. The recombinant construct was treated with and without CpG methyltransferase (methylated and unmethylated) and transfected into A549, HEK293 and HT29 cells. At 24 h posttransfection, the luciferase activity was measured; the luciferase activity is presented relative to that of the empty vector. The experiments were performed in triplicate, and the data are presented as the mean ± S.E.M. **P*<.05 and ***P*<.01 compared with the unmethylated construct. (B) The relative expression of miR-9-3 was quantified by qPCR. ***P*<.01 compared with the control.

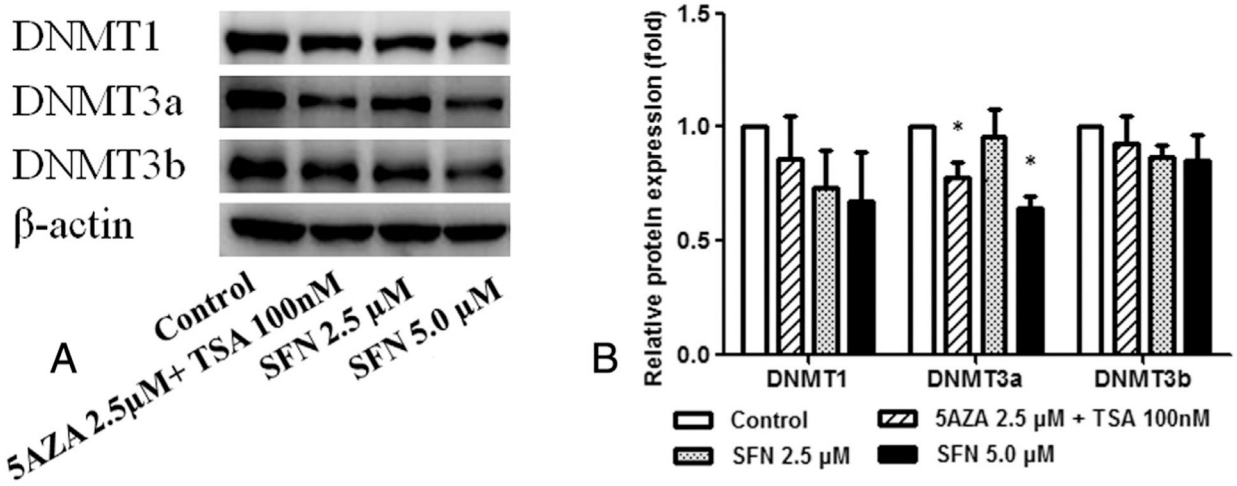

Fig. 4.

SFN decreases the protein levels of DNMTs. The DNMT protein levels were determined by Western blotting (A). Relative expression was calculated by measuring the intensity of each band and normalizing each value to that of β-actin (B). **P*<.05 compared with the control.

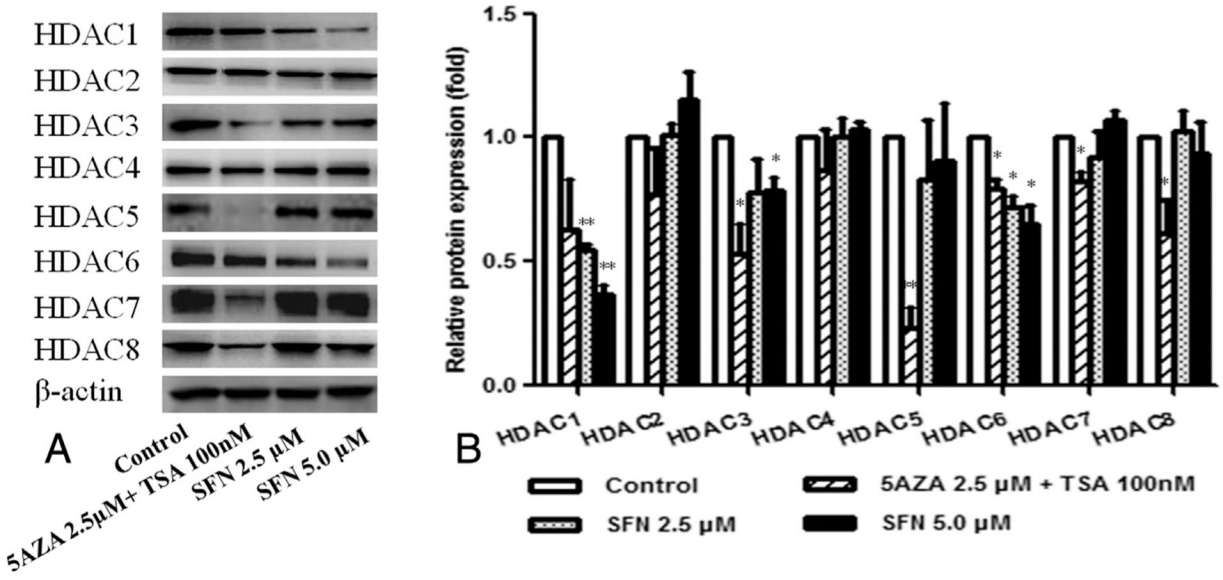

Fig. 5.

SFN reduces the protein levels of HDACs. The HDAC protein levels were determined by Western blotting (A). Relative expression was calculated by measuring the intensity of each band and normalizing each value to that of β-actin (B). **P*<.05 and ***P*<.01 compared with the control.

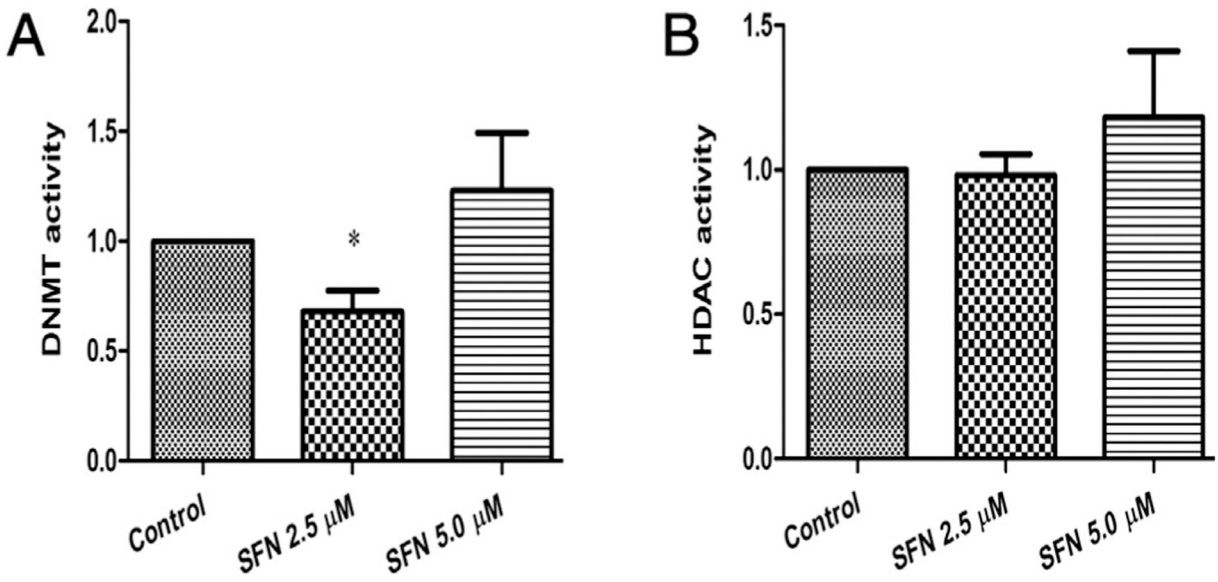

Fig. 6.

SFN diminishes DNMT activity. The relative activity of DNMT (A) and HDAC (B) was calculated as the ratio of enzymatic activity in the SFN treatment group to that in the control group. **P*<.05.

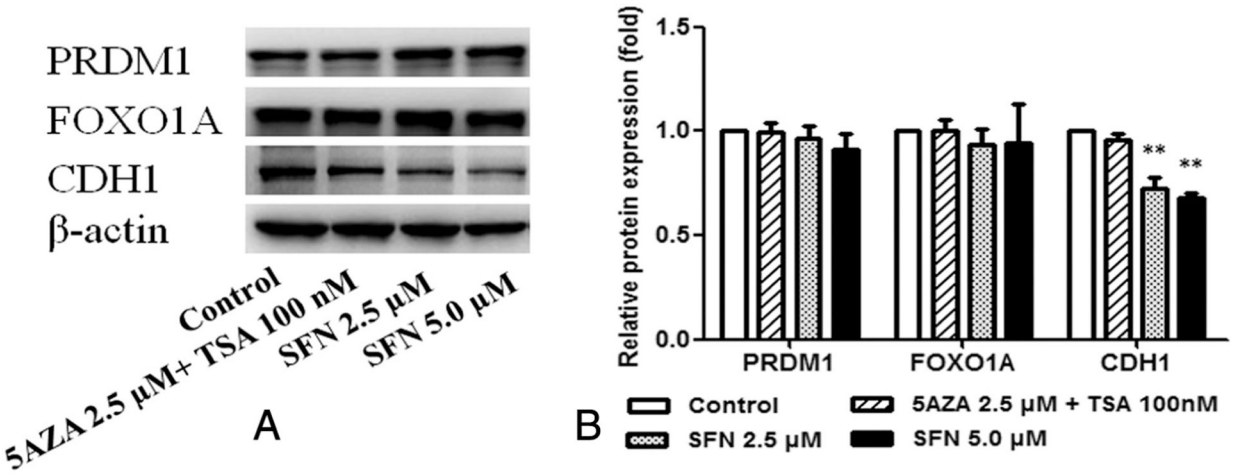

Fig. 7.

SFN decreases the protein levels of CDH1. The protein levels of miR-9-3 target genes (i.e., *PRDM1*, *FOXO1A* and *CDH1*) were determined by Western blotting (A). Relative expression was calculated by measuring the intensity of each band and normalizing each value to that of β-actin (B). ** $P<.01$ compared with the control.