Sulforaphane-induced epigenetic regulation of Nrf2 expression by DNA methyltransferase in human Caco-2 cells

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Abstract. The present study aimed to investigate the mechanism underlying sulforaphane-mediated epigenetic regulation of nuclear factor-erythroid derived 2-like 2 (Nrf2) expression in human colon cancer. Proteins were extracted from normal Caco-2 cells using sulforaphane and 5-aza-2'-deoxycytidine (5-Aza) combined with trichostatin A (TSA). The mRNA and protein expression levels and activity of DNA methyltransferase 1 (DNMT1) were determined. Methylation-specific polymerase chain reaction and bisulfite genomic sequencing were also used to measure the methylation levels of CpG sites in the Nrf2 promoter region. Nrf2 expression was measured using reverse transcription-quantitative PCR and western blot analysis. The results demonstrated that sulforaphane did not affect DNMT1 mRNA expression levels. DNMT1 protein expression was inhibited by sulforaphane and 5-Aza co-treatment with TSA. Nrf2 promoter methylation decreased significantly in the sulforaphane group compared with the control group. Nrf2 promoter methylation level in the 5-Aza+TSA group was the lowest among all groups. Nrf2 mRNA levels exhibited significant differences between the sulforaphane-treated and control groups, as well as between the 5-Aza+TSA and control groups, and the sulforaphane-treated and 5-Aza+TSA groups. Nrf2 protein expression was also inhibited by sulforaphane, as well as 5-Aza co-treatment with TSA. The results revealed that sulforaphane may promote demethylation of the Nrf2 promoter region to increase activation of Nrf2, which induces chemoprevention of colon cancer.

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Abbreviations: 5-Aza, 5-aza-2'-deoxycytidine; TSA, trichostatin A; DNMT1, DNA methyltransferase 1; MSP, methylation-specific PCR; BGS, bisulfite genomic sequencing; UGT, UDP-glucuronosyltransferase; sMaf, small Maf protein; Nrf2, nuclear factor-erythroid derived 2-like 2; Keap1, Kelch-like ECH-associated protein 1

Key words: sulforaphane, chemoprevention, colon tumour, nuclear factor-erythroid derived 2-like 2, epigenetic regulation

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide. The estimated incidence of CRC is 10.2% and ranks third among all of the cancer types globally. The mortality of CRC is 9.2% and ranks second all over the world (1). Effective early diagnosis of colon cancer is limited, and thus, colon cancer is diagnosed in the middle or late stage. The treatment of colon cancer is relatively passive (2,3). Therefore, the prevention of colon cancer is very important for public health.

Environmental factors, including high-fat, high-protein and low-fibre diets, are partially accountable as causes of colon cancer. The consumption of processed meat and alcohol are also associated with CRC (4). Whole grains, dietary fibre, dairy products and red meat may influence the oncogenesis of CRC (4). This suggests that an individual's diet is an important factor in the pathogenesis of CRC.

A number of natural products have anti-CRC effects, including honey, bee pollen, propolis and *Brassicaceae* extracts (5,6). The present study focused on the effect of sulforaphane, a phytochemical extracted from *Brassicaceae.*

Heterocyclic amines produced in meat cooked at high-temperatures are associated with colon carcinogenesis. These amines are oxidised to N-hydroxy metabolites by cytochrome P450 family 1 subfamily A member 2 oxidase in the liver and migrate into the intestinal mucosa through the blood. Following N-acetyl transferase acetylation in intestinal epithelial cells, the heterocyclic amines bind to DNA and form DNA adducts, which may cause chromosome translocation, cancer-related gene mutations, microsatellite instability and chain mutations, eventually leading to colon cancer (7,8). UDP-glucuronosyltransferase (UGT) is a phase II enzyme that catalyses the metabolism of heterocyclic amines, including the glucuronic acid conjugation reaction that removes DNA adducts.

Classic UGT inducers are often toxic. However, phytochemicals have been demonstrated to exhibit preventive effects against cancer in animal experiments and epidemiological

studies. Phytochemicals can directly remove environmental carcinogens and induce cell phase II enzymes, which increases the metabolism and removal of carcinogens (9).

Multiple mechanisms have been identified for the cancer-associated chemo-preventive activities of sulforaphane. Sulforaphane is a potent monofunctional inducer of phase II enzymes, as demonstrated by studies using cultured cells, mouse tissues (10,11), human intestine (12) and human airways (13). Sulforaphane was also tested in humans and was revealed to improve hepatic abnormalities (14). Sulforaphane induced phase II detoxification enzymes, such as UGT (8). It also inhibited three cytochrome P450 isoforms (CYP1A1, CYP2B1/2 and CYP3A4) (15). Additionally, sulforaphane slowed the cell cycle progression of a prostate cancer cell line LNCaP and induced apoptosis in human glioblastoma T98G and U87MG cells (16,17). In addition, sulforaphane inhibited the initiation of carcinogen-induced skin tumours (18,19), and reduced metastatic spread of melanoma in mice (20). This chemical also promoted the antiproliferative activity of other antiproliferative agents, including oxaliplatin (21).

Sulforaphane, which is produced by cruciferous vegetable plants, has been demonstrated to inhibit or retard tumour incidence and progression in models of breast, colon, stomach and lung cancer (22). The molecular mechanism of the effect of sulforaphane on colon cancer is partly understood; sulforaphane acts by multiple pathways including the inhibition of inflammatory cytokine production (23) and downregulation of nuclear factor (NF)-κB activity (24). However, there are few studies on the epigenetic regulation of gene expression in colon cancer cells by sulforaphane (21).

Nuclear factor-erythroid derived 2-like 2 (Nrf2) is a leucine zipper transcription factor, which serves an important role in the maintenance of redox balance and cytoprotection against chemical carcinogens (25,26). Under oxidative and electrophilic stress conditions, Nrf2 is released by Kelch-like ECH-associated protein 1 (Keap1)-mediated rapid degradation. Nrf2 is stabilised, accumulated and translocated to the nucleus, where it dimerises with a small Maf protein (sMaf). The Nrf2-sMaf heterodimer binds to a specific DNA sequence, referred to as the antioxidant/electrophile response element (ARE/EpRE), and induces the expression of cytoprotective enzymes (11,25,26).

In our previous studies, sulforaphane was demonstrated to activate the transcription and expression of the UDP glucuronosyltransferase family 1 member A complex locus (UGT1A) gene via Nrf2 (27). Curcumin also induces Nrf2 expression by demethylating five CpG loci in the promoter region of the Nrf2 gene (28). The epigenetic regulatory effect of sulforaphane has been identified in a number of types of tumours, including its ability to inhibit DNA methyltransferases (DNMTs) in prostate cancer (29). Sulforaphane also functions as a histone deacetylase inhibitor to regulate gene expression (28,30,31).

In the present study, it was hypothesised that sulforaphane may inhibit DNMT to induce the demethylation of CpG sites in the Nrf2 promoter region and increase the expression of Nrf2 in Caco-2 cells. The expression and activity of DNMT1, methylation-specific polymerase chain reaction (MSP) and bisulfite genomic sequencing (BGS) of Nrf2 and the differences between the epigenetic regulation of sulforaphane and 5-aza-2'-deoxycytidine (5-Aza) combined with trichostatin A (TSA) were examined to determine the epigenetic regulation of sulforaphane on Nrf2 expression in human colon cancer. The combined use of DNMT inhibitor 5-aza and the histone deacetylase (HDAC) inhibitor TSA has been demonstrated to be able to reverse epigenetic modifications and increase the expression of NRF2 and its downstream antioxidant and detoxification enzymes (32).

Materials and methods

Reagents. Sulforaphane (Sigma-Aldrich; Merck KGaA) was dissolved to 1 µmol/ml in dimethyl sulfoxide (DMSO) and stored at -20˚C for further use. TSA and 5-Aza were purchased from Sigma-Aldrich.

Cell culture. Caco-2 human colon adenocarcinoma cells (Shanghai Institutes for Biological Science, Chinese Academy of Sciences) were incubated as monolayers in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% heat-inactivated foetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C in a humidified incubator with 5% CO_2. Caco-2 cells were selected as they are enterocyte-like and appropriate for the testing of pharmacologically active molecules generated in drug discovery programmes, which suited the aim of the present study (33,34). Cells in the logarithmic growth phase were used for the experiments. A comparison between Caco-2 cells cultured in DMEM and DMEM supplemented with the solvent of sulforaphane solution, DMSO, at a concentration of <0.1%, was performed, and no difference was observed, which revealed that DMSO was not toxic to Caco-2 cells.

Drug treatment. There were three groups, the control group, sulforaphane-treated group and 5-Aza+TSA group. Caco-2 cells were cultured in 6-well plates (40,000 cells/well) at 37˚C and 5% CO_2 for 24 h. As the cells reached 70-80% confluence, the medium was replaced. Based on the results of our previous study, which demonstrated that the expression of UGT1A protein was induced in a dose-dependent manner following 24-h treatment with 10-30 µmol/l sulforaphane, and this induction by sulforaphane was most powerful at 25 µmol/l (35), 25 µmol/l sulforaphane treatment was selected to test whether the pathways influencing the effect of sulforaphane to UGT1A include epigenetic changes of Nrf2. In the control group, cells were cultured in 2 ml medium with 0.1% DMSO for 24 h. In the sulforaphane-treated group, 2 ml of complete medium with 25 µmol/l of sulforaphane was added, and the cells were cultured for 24 h. A total of 2 ml of medium with 5-Aza (10 µmol/l) and TSA (1 µmol/l) was added to the 5-Aza+TSA group and then incubated for 24 h. After incubation the cells were used in the processes below.

DNA extraction and C-T conversion. DNA was extracted from all three groups using Total DNA extraction kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. Briefly, cell lysis buffer A (200 µl) and proteinase K (20 µl) were added into the cell culture. The mixture was stirred for 5 min at 25˚C and buffer B (200 µl) was added. The solution was mixed by inversion and incubated at 70˚C for 10 min. A

Table I. Primers for BGS and MSP to target the first five CpGs of the murine Nrf2 gene.

Experiment		Primer sequence, 5'-3'
BGS	F:	GGTTTTGTAATTTTAAATTAGGGAGG
	R:	ACAACTCCAAATCCATCATAATAAAC TATA
MSP	F1:	GTTTTAAAGCGTTCGAATTTTAGC
	R1:	GTTAACTCCCCGATACCGAC
	F2:	TCGTTTTCGGATCGCGAG
	R2:	GCGACGCGAACAAAACG
USP	F1:	TGTTTTAAAGTGTTTGAATTTTAGTGA
	R1	TCCATTAACTCCCCAATACCAA
	F2:	TTGTTTTTGGATTGTGAGTTTTTTG
	R2:	CAAAACACAACACAAACAAAACA ACT

BGS, bisulfite genomic sequencing; MSP, methylation-specific PCR; UGT, UDP-glucuronosyltransferase; F, forward; R, reverse.

Table II. Primers for RT-qPCR.

Gene		Primer sequence, 5'-3'
GAPDH	F:	CATGAGAAGTATGACAACAGCCT
	R:	AGTCCTTCCACGATACCAAAGT
NFE2L2	F:	CAAGAGAAAGCCTTTTTCGCTCAG
	R:	GAATGTGGGCAACCTGGGAGTAG
UGT1A10	F:	ACTGTCATCAGGGAAAGCCATTG
	R:	CACAATTCCATGTTCTCCAGAAGC

F, forward; R, reverse.

spin column was used to collect DNA. Following centrifugation, washing and drying, total DNA was obtained and eluted with 30 μl elution buffer.

Zymo EZ DNA Methylation-Gold™ Kit (Zymo Research Corp.) was used for C-T conversion to separate methylated cytosines from unmethylated uracils. First, a C-T conversion reagent was used for the sulfonation of unmethylated DNA. During this reaction, the tube was incubated at 98˚C for 10 min and at 64˚C for 2.5 h. To collect and separate the DNA with a Zymo-spin IC column, M-binding buffer (600 μl) was used for hydrolytic deamination. During the procedure, M-desulfonation (200 μl) buffer was used, followed by M-wash buffer (200 μl) and M-elution buffer (10 μl).

BGS. BGS primers were designed based on the DNA sequences that were enriched in CpG islands in the promoter region of the Nrf2 gene (Table I). Primers were designed to target the first five CpGs of the murine Nrf2 gene (from -255 to -70 bp). The PCR reaction mixture included DNA (2 μl), ddH_2O (6 μl), forward primer (1 μl), reverse primer (1 μl) and 10 μl Go Taq® Green Master Mix (Promega Corporation). The thermocycling conditions were as follows: 95˚C for 10 min, followed by 95˚C for 30 sec, 54˚C for 30 sec, and 72˚C for 40 sec for 40 cycles and 72˚C for 10 min. Subsequently, 6X DNA loading buffer (Invitrogen; Thermo Fisher Scientific, Inc.), and PCR products were mixed with 10X GelRed Nucleic Acid Stain (Biotium, Inc.). Agarose gel electrophoresis (1.2%) was performed under 120 V. A gel digital imaging system (Gel Doc XR+; Bio-Rad Laboratories, Inc.) was used for colour rendering. The PCR gel was then cut under UV light, and Zymoclean Gel DNA Recovery kit (Zymo Research Corp.) was then used for recovery of DNA according to the manufacturer's protocols.

The recovered DNA products were used in TA cloning, and the reaction mixture was as follows: PCR products (3 μl), T4 ligase (1 μl; Thermo Fisher Scientific, Inc.), pGEM®-T Easy Vector (1 μl; Promega Corporation) and 2X ligation buffer (5 μl). The resulting product was incubated at 4˚C overnight. Subsequently, 10 μl reaction mixture was added to 100 μl of DH5 competent *E. coli* (Thermo Fisher Scientific, Inc.), placed on ice for 30 min and then activated at 42˚C for 90 sec. The mixture was stirred at 0˚C for 2 min, 1 ml lysogeny broth (LB; Thermo Fisher Scientific, Inc.) liquid medium without antibody was added, and the mixture was stirred at 37˚C for 1 h. The recovered bacteria were centrifuged at 503.1 x g for 2 min. Subsequently, 200 μl of bacteria suspension was mixed with X-gal (Biovision, Inc.) and isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich; Merck KGaA), evenly coated on LB solid medium with ampicillin and cultured at 37˚C overnight. A total of 10 monoclonal colonies were selected from each group and inoculated into 3 ml of liquid medium containing ampicillin at 37˚C overnight. The resulting bacteria were sent to a third party (Biozeron) for sequencing the next day.

MSP. Using DNA samples with C-T conversion, methylated DNA was amplified using specifically designed primers (Table I) targeting the first five CpGs of the Nrf2 gene (from -313 to -166 bp). The PCR reaction mixture was as follows: DNA (2 μl), ddH_2O (6 μl), forward primer (1 μl), reverse primer (1 μl) and Go Taq® Green Master Mix (10 μl). The thermocycling conditions were as follows: an initial denaturation at 95˚C for 10 min; 40 cycles of 95˚C for 30 sec, 54˚C for 30 sec and 72˚C for 40 sec for amplification; and a final extension at 72˚C for 10 min. PCR products were subjected to 2% agarose gel electrophoresis at 120 V, and visualised under UV light.

Reverse transcription-quantitative PCR (RT-qPCR). After drug treatment as aforementioned, total RNA was isolated from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcriptase reactions were performed using 1 μl RNA, primers (Table II) and M-MLV reverse transcriptase (Toyobo Life Science) with a final volume of 20 μl. The presence of Nrf2 and DNMT1 transcripts was analysed by qPCR with SYBR-Green (Toyobo Life Science), using the Agilent Stratagene 3000P RT-qPCR instrument (Agilent Technologies, Inc.). The expression level of the housekeeping gene GAPDH was used as an internal control. The following thermocycling conditions were used for the PCR: 95˚C for 3 min; 40 cycles of 95˚C for 12 sec and 62˚C for 40 sec. Measurements were conducted in triplicate. The relative amount of mRNA was calculated by the $2^{-\Delta\Delta Cq}$ method (36). All primers were synthesised by Shanghai BioSun Sci&Tech Co., Ltd. using the Basic Local Alignment Search Tool (http://www.urogene.org/methprimer/). Gene-specific amplifications were determined

by analysing RT-qPCR product bands following agarose gel electrophoresis and melting curve data.

Western blot analysis. Following the aforementioned drug treatments, Caco-2 cells were washed twice with ice-cold PBS and lysed in complete cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM NaF, 1 mM dithiothreitol, 1 mM PMSF, 1 mM activated Na_3VO_4, 0.02 μM aprotinin, 0.16 μM leupeptin and 0.22 μM pepstatin). Bicinchoninic acid assay was used to determine protein concentration in cell lysates. Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. A Trans-Blot® semi-dry transfer cell (Bio-Rad Laboratories, Inc.) was used for semi-dry electrophoretic transfer at 30 mA for 90 min. The membranes were incubated with blocking buffer (5% milk powder dissolved in TBST) at 4˚C overnight. Membranes were probed with primary monoclonal antibodies against GAPDH (cat. no. AM4300; 1:2,000; Thermo Fisher Scientific, Inc.), Nrf2 (cat. no. ab62352; 1:1,000; Abcam) and DNMT1 (cat. no. ab134148; 1:1,000; Abcam) at 4˚C overnight. Following washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. D110058-0100; 1:5,000; Sangon Biotech Co., Ltd.) at 37˚C for 2 h. The bands were detected by enhanced chemiluminescence. The intensities of acquired bands were measured by Gel-Pro Analyzer computerised image analysis system and normalised to GAPDH as the endogenous control.

DNMT1 enzyme activity detection. A TaqMan® Array Human DNA Methylation and Transcriptional Repression 96-well plate standard kit (Epigentek Group Inc.) was used according to the manufacturer's protocol to determine the activity of DNMT1 in Caco-2 cells following drug treatment. The inhibitory rate was calculated as follows: DNMT1 inhibitory rate (%)=1-[sample (OD450)-blank (OD450)]/[control (OD450)-blank (OD450)].

Statistical analysis. Statistical analysis was performed by one-way ANOVA using SPSS 22.0 (IBM Corp.). Data are presented as the mean ± SD. Differences among different treatment groups were analysed using the Student-Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Sulforaphane does not induce DNMT1 mRNA expression in Caco-2 cells. DNMT1 mRNA expression in Caco-2 cells was tested following treatment with sulforaphane or co-treatment with 5-Aza and TSA. DNMT1 mRNA expression in sulforaphane-treated samples was not significantly different compared with the control group. However, DNMT1 mRNA expression level was significantly reduced in the 5-Aza+TSA group compared with the control and the sulforaphane-treated groups (Fig. 1A).

Sulforaphane induces Nrf2 mRNA expression in Caco-2 cells. The induction of Nrf2 expression by sulforaphane was measured using RT-qPCR. The Nrf2 mRNA level in the sulforaphane-treated group and 5-Aza+TSA group were significantly increased compared with the control group (Fig. 1B). The mRNA level of Nrf2 in 5-Aza+TSA group was significantly increase compared with sulforaphane-treated group (Fig. 1B). The RT-qPCR products were confirmed by the amplification plots and the dissociation curve (Fig. 1C-F).

Sulforaphane inhibits DNMT1 protein expression in Caco-2 cells. The DNMT1 protein levels in Caco-2 cells from the control, sulforaphane-treated and 5-Aza+TSA groups were compared. Western blot analysis revealed that DNMT1 protein expression was reduced by sulforaphane and by 5-Aza combined with TSA (Fig. 2A and B).

Sulforaphane increases Nrf2 protein in Caco-2 cells. Nrf2 protein levels were compared between the control group, sulforaphane-treated group and 5-Aza+TSA group. Western blot semi-quantitative grey analysis revealed that Nrf2 protein expression increased following treatment with sulforaphane or 5-Aza combined with TSA (Fig. 2C and D).

Sulforaphane decreases methylation in the promoter region of Nrf2 gene in Caco-2 cells. BGS revealed that the promoter region of the Nrf2 gene had high levels of DNA methylation (Fig. 3A and B). Following sulforaphane or 5-Aza+TSA treatment, Nrf2 promoter methylation level decreased significantly compared with the control group. The 5-Aza+TSA group exhibited a greater decline (Fig. 3B). These results suggested that sulforaphane or 5-Aza+TSA treatment may upregulate Nrf2 expression by reducing the methylation of its promoter region. Compared with the control group, MSP and USP results demonstrated no differences between the sulforaphane-treated, the 5-Aza+TSA, and the control groups, which indicated that following sulforaphane or 5-Aza+TSA treatment, the methylation of the Nrf2 gene promoter in Caco-2 cells did not change significantly (Fig. 3D). A possible explanation is that the MSP and USP primers were not sufficiently sensitive or that demethylation does not occur in this section.

Sulforaphane inhibits DNMT1 protein activity. The result of DNMT1 enzyme activity detection demonstrated that DNMT1 activity was significantly inhibited in the sulforaphane-treated group and the 5-Aza+TSA-treated group compared with the control group, and there was significant difference between the sulforaphane-treated and the 5-Aza+TSA-treated groups (Fig. 3C).

Discussion

Epigenetic silencing through hypermethylation of the promoter area is involved in transcriptional repression of growth regulatory genes and numerous tumour suppressor genes in cancer cells (37). The balance of these processes is regulated by many types of molecules (38), including DNMT, HDACs and Keap1 enzymes, the disruption of which contributes to carcinogenesis. Studies have demonstrated that sulforaphane serves an important role in inducing methylation changes in cancer cells as a DNMT inhibitor (39,40). Antiproliferative, antioxidant and apoptosis-inducing effects of sulforaphane have been demonstrated in many studies (22,41) on several cancer types, including CRC. However, the detailed molecular mechanisms of sulforaphane actions remain to be elucidated. Previous

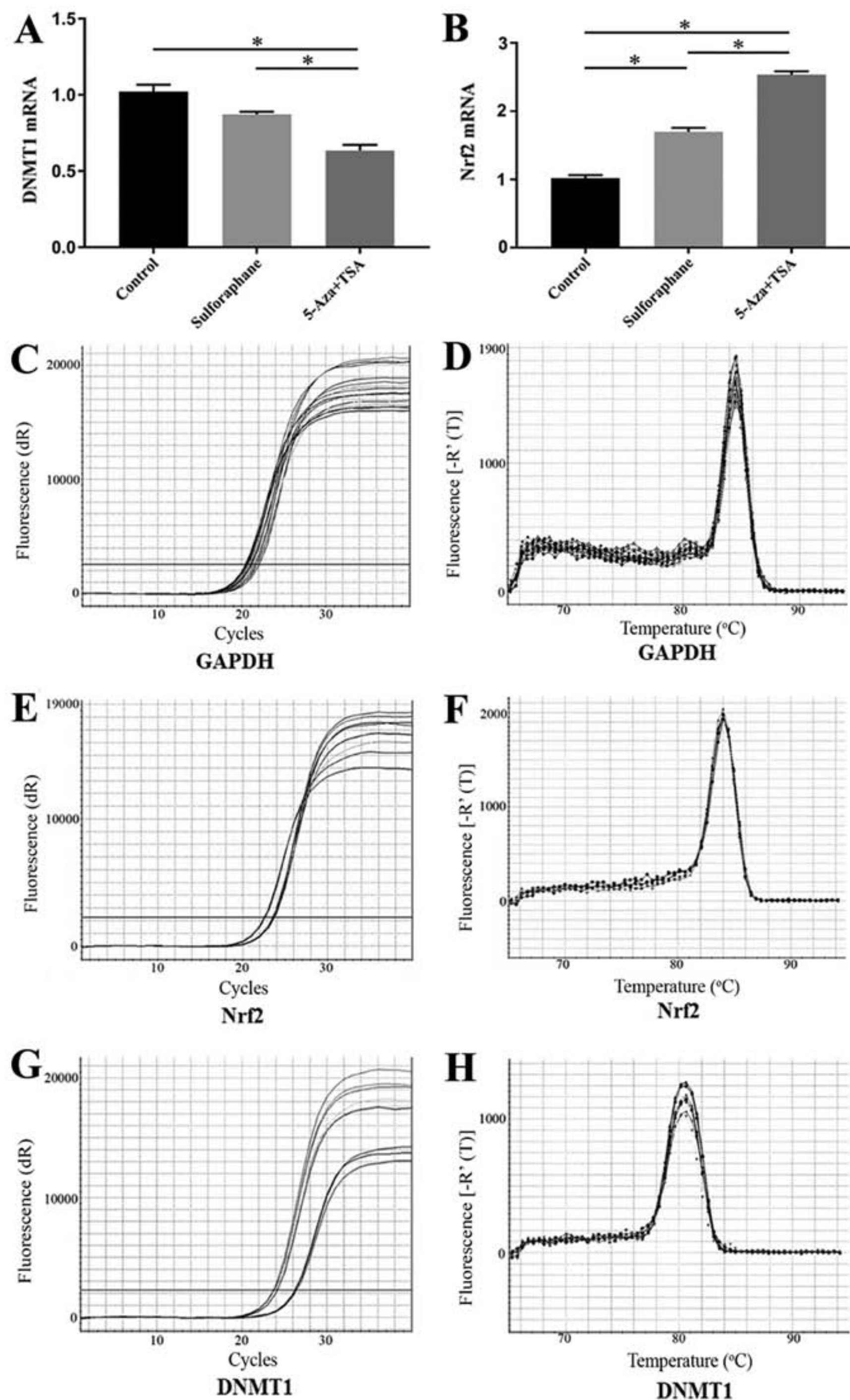

Figure 1. Effects of sulforaphane and 5-Aza co-treatment with TSA on DNMT1 and Nrf2 mRNA expression. (A) DNMT1 expression levels in Caco-2 cells treated with sulforaphane or 5-Aza and TSA. (B) Nrf2 mRNA expression in Caco-2 cells treated with sulforaphane or 5-Aza and TSA. (C) Amplification plots and (D) dissociation curves of GAPDH. (E) Amplification plots and (F) dissociation curves of Nrf2. (G) Amplification plots and (H) dissociation curves of DNMT1. *P<0.05. 5-Aza, 5-aza-2'-deoxycytidine; Nrf2, DNMT1, DNA methyltransferase 1; nuclear factor-erythroid derived 2-like 2; RT-qPCR, reverse transcription-quantitative PCR; TSA, trichostatin A.

studies have demonstrated that sulforaphane impacts global DNA methylation and site-specific demethylation (31,39). This suggests that the DNA methylation alteration of specific oncogenesis-controlling genes may be important in CRC chemoprevention.

Our previous study demonstrated that the expression and activity levels of UGT1A in CRC tissues were lower compared with normal tissues (42). The human colon exhibits a complex pattern of UGT1A loci expression, with UGT1A8 and UGT1A10 predominantly expressed in the colon (43). Regulation of the UGT1A gene is affected by a polymorphic region in colonic mucosal epithelium, and different individuals have different susceptibilities to carcinogens as a result of differential UGT1A expression (35,43). Decreased activity of UGT1A and its isoforms UGT1A8 and UGT1A10 may be a factor in the pathogenesis of CRC (43). Our previous study revealed that sulforaphane in low doses induced UGT1A, 1A8 and 1A10 mRNA expression. UGT1A protein expression

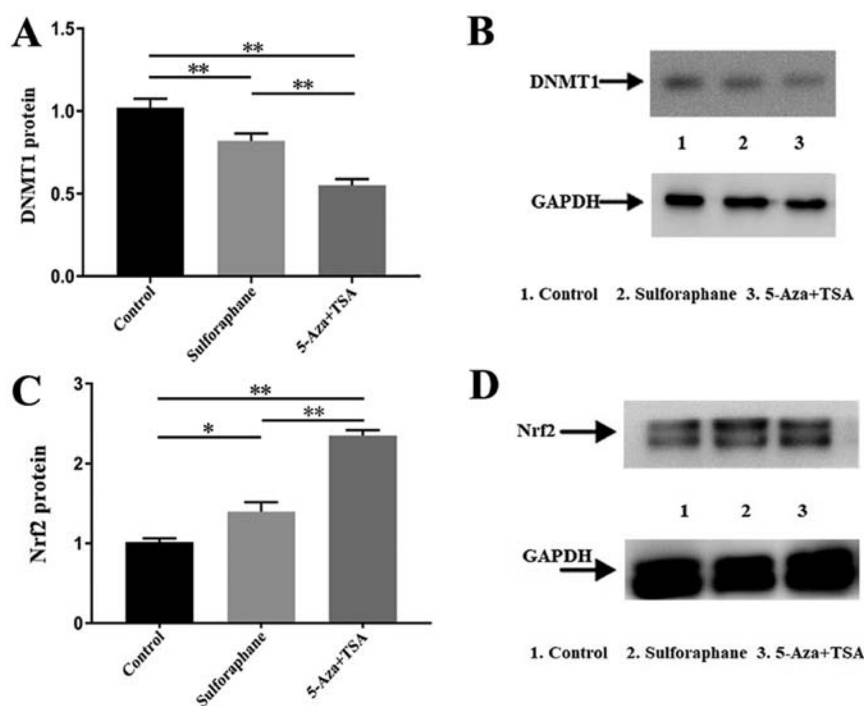

Figure 2. Sulforaphane reduces DNMT1 and increases Nrf2 protein expression in Caco-2 cells. (A) Determination of DNMT1 expression by semi-quantitative grey analysis. (B) Western blotting images of DNMT1 expression relative to GAPDH. (C) Determination of Nrf2 expression by semiquantitative grey analysis. (D) Western blotting images of Nrf2 protein expression. Images are representative of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$. 5-Aza, 5-aza-2'-deoxycytidine; DNMT1, DNA methyltransferase 1; TSA, trichostatin A.

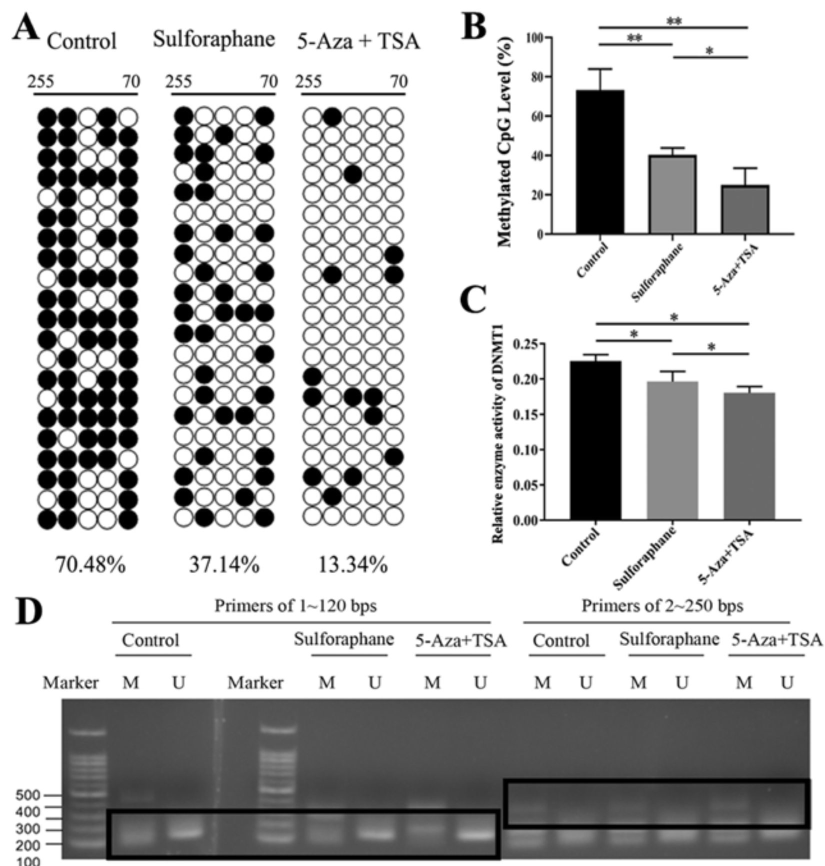

Figure 3. BGS and MSP of the promoter region of the Nrf2 gene. The result is representative of three independent experiments. (A) DNA methylation of the Nrf 2 promoter as determined by BGS; black spots denote methylated CpGs and white dots denote unmethylated CpGs; percentages indicate the proportion of CpGs that are methylated. (B) Quantification of Nrf2 promoter methylation based on BGS. (C) Relative enzyme activity of DNMT1 in Caco-2 cells treated with sulforaphane or 5-Aza and TSA. (D) MSP of the promoter region of Nrf2 n Caco-2 cells treated with sulforaphane or 5-Aza and TSA. $^{*}P<0.05$, $^{**}P<0.01$. 5-Aza, 5-aza-2'-deoxycytidine; TSA, trichostatin A; M, methylated; U, unmethylated; BGS, bisulfite genomic sequencing; MSP, methylation-specific PCR.

increased the glucuronic acid binding capacity of heterocyclic amines (35). Therefore, sulforaphane may activate the transcription of UGT1A and increase Nrf2 expression.

The epigenetic mechanisms of the anticancer activity of sulforaphane have been partly identified by previous studies, which have indicated that sulforaphane acts through histone acetylation, histone phosphorylation, DNA methylation and non-coding RNA regulation (30,39). Sulforaphane has been demonstrated to inhibit cancer cell transformation and development by CpG demethylation at the Nrf2 promoter in TRAMP C1 prostate cancer cells (32). The aim of the present study was to explore whether colon cancer cells are affected by sulforaphane in a similar manner.

In the present study, the human colon cancer cell line Caco-2 was cultured with 25 μM sulforaphane or 5-Aza combined with TSA. The concentration of sulforaphane used was determined based on a previous study (35). The results of the present study demonstrated that sulforaphane may inhibit DNMT1 expression and reduce its activity by demethylating the promoter region of Nrf2 and increasing Nrf2 expression.

The expression and activity of DNMT1 were measured to confirm whether DNMT1 in Caco-2 cells was affected by sulforaphane. The RT-qPCR results demonstrated that the DNMT1 mRNA expression was not affected by sulforaphane but significantly reduced by 5-Aza combined with TSA. This result revealed that the effects of sulforaphane on DNMT1 mRNA transcription are less potent compared with 5-Aza+TSA, and the differences between the sulforaphane and the control groups require further studies. A significant decrease of DNMT1 mRNA expression has been demonstrated in LnCap prostate cancer cells after sulforaphane treatment (31). This may be due to metabolic and oncogenic differences between prostate and colon cancer.

Western blot analysis demonstrated that there were significant differences between each group in DNMT1 protein expression. A reduction in DNMT1 protein expression has also been reported in LnCap and TRAMP C1 cells (31,32), which revealed that sulforaphane may decrease DNMT1 protein expression in colon and prostate cancer. In the present study, the mRNA level of DNMT1 in the sulforaphane-treated group and the control group were not significantly different while the protein level was significantly different, indicating that the expression process may be blocked or weakened by other pathways that were not investigated. The present results demonstrated that sulforaphane may reduce DNMT1 protein expression, and therefore, may help demethylate the promoter region of Nrf2 through this pathway, similar to other enzymes, including HDAC (10,31). The effects of sulforaphane were lower compared with 5-Aza combined with TSA.

The results of the present study demonstrated that Nrf2 transcription and expression were significantly increased following treatment with either sulforaphane or 5-Aza+TSA treatment. In present study, the effect of 5-Aza+TSA on DNMT1 and Nrf2 expression was greater compared with the effect of sulforaphane on Nrf2 expression. The difference may be a result of the different treatment concentrations, treatment time, mechanism of action or cellular resistance. 5-Aza+TSA treatment can strongly inhibit DNMT1 and HDAC through proteins and noncoding miRNAs, causing genome-wide hypomethylation resulting in the expression of several tumour suppressor genes causing growth arrest of cancer cells (44). Further studies are required to understand the differences between 5-Aza+TSA and sulforaphane. A study by Zhang *et al* (32) reported a similar result in prostate cancer cells: Following sulforaphane treatment, mRNA and protein expression of Nrf2 was significantly induced in TRAMP C1 cells. Therefore, sulforaphane may induce Nrf2 activation in more than one type of cancer cells. However, the most effective concentrations and incubation times are different for the two types of cancer cells.

To identify potential DNA methylation changes mediated by sulforaphane, BGS and MSP were performed. BGS revealed that following sulforaphane or 5-Aza+TSA treatment, Nrf2 promoter methylation decreased significantly compared with the control group; the 5-Aza+TSA group exhibited the greatest decline. This suggested that either sulforaphane or 5-Aza+TSA treatment may upregulate Nrf2 expression by reducing the methylation level of the Nrf2 promoter region, and 5-Aza+TSA treatment had a stronger effect. Another study demonstrated that sulforaphane may decrease the methylated CpG ratio in the promoter region of Nrf2 gene in TRAMP C1 cells (36). The MSP experiment did not exhibit the same trend, possibly because the MSP and USP primers were not sufficiently sensitive. This issue needs to be addressed by further studies. The activity of DNMT1 protein was significantly decreased in the sulforaphane-treated group and the 5-Aza+TSA-treated group compared with controls, indicating that the function of DNMT1 protein may be inhibited, which was not demonstrated in previous studies. These results indicated that sulforaphane may induce demethylation of the promoter area of Nrf2. However, sulforaphane may have a systemic demethylation-inducing effect that could impact the epigenetic stability of the gene. Therefore, sulforaphane may cause harmful side effects throughout the human body as the specificity or targets of the effect of sulforaphane on methylation changes are widely distributed all over the body in cancer cells as well as normal cells. A relatively high concentration of sulforaphane is required to induce significant methylation changes compared to the amount we consume daily, so it is important to evaluate the safety and reliability of high doses. Future studies employing additional colon cancer cell lines are required to increase the reliability of these results.

In summary, the present study demonstrated that through epigenetic regulation, sulforaphane may inhibit DNMT1 protein expression and reduce DNMT1 activity, which may lead to the demethylation of the promoter region of Nrf2 and increased activation of Nrf2, inducing the transcription of the defensive enzymes UGTs, and leading to homeostatic protection of cells and tissues against exogenous and/or endogenous carcinogens. The results of the present study demonstrated that although sulforaphane had a weaker effect than 5-Aza combined with TSA, it could serve an important role in colon cancer prevention through the demethylation-inducing pathway. Further studies are necessary to confirm the nuclear translocation pathway induced by sulforaphane. In addition, future studies should explore the commercial value and pharmacological mechanism of sulforaphane, which may help advance the commercialisation of this chemical.

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Availability of data and materials

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

Authors' contributions

JWZ designed, performed the research and wrote the manuscript. MW designed and supervised the study. YQ, TFY, CL, DW and NXS contributed to the methylation-specific polymerase chain reaction and bisulfite genomic sequencing experiments and analysis. All authors confirm the accuracy of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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