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DNA methylation regulates expression of VEGF-C, and S-adenosylmethionine is effective for VEGF-C methylation and for inhibiting cancer growth

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Abstract

DNA hypomethylation may activate oncogene transcription, thus promoting carcinogenesis and tumor development. S-adenosylmethionine (SAM) is a methyl donor in numerous methylation reactions and acts as an inhibitor of intracellular demethylase activity, which results in hypermethylation of DNA. The main objectives of this study were to determine whether DNA hypomethylation correlated with vascular endothelial growth factor-C (VEGF-C) expression, and the effect of SAM on VEGF-C methylation and gastric cancer growth inhibition. VEGF-C expression was assayed by Western blotting and RT-qPCR in gastric cancer cells, and by immunohistochemistry in tumor xenografts. VEGF-C methylation was assayed by bisulfite DNA sequencing. The effect of SAM on cell apoptosis was assayed by flow cytometry analyses and its effect on cancer growth was assessed in nude mice. The VEGF-C promoters of MGC-803, BGC-823, and SGC-7901 gastric cancer cells, which normally express VEGF-C, were nearly unmethylated. After SAM treatment, the VEGF-C promoters in these cells were highly methylated and VEGF-C expression was downregulated. SAM also significantly inhibited tumor growth *in vitro* and *in vivo*. DNA methylation regulates expression of VEGF-C. SAM can effectively induce VEGF-C methylation, reduce the expression of VEGF-C, and inhibit tumor growth. SAM has potential as a drug therapy to silence oncogenes and block the progression of gastric cancer.

Key words: VEGF-C; Methylation; S-adenosylmethionine; Gastric cancer

Introduction

Gastric cancer is a common digestive-system malignancy, especially in East and Southeast Asia (1). Gastric cancer accounts for more than 10% of cancer deaths worldwide, second only to lung cancer (2). Most patients are diagnosed at advanced stages, typically with a poor prognosis, 5-year survival rates of $<$30%, metastasis, and relapse (3). Vascular endothelial growth factor-C (VEGF-C) is secreted by most solid tumors including gastric cancer. Strong expression of VEGF-C is considered an important predictor of lymphangiogenesis and as a prognostic marker in numerous types of cancers, including gastric carcinoma (4-6). VEGF-C is considered to be a novel mediator of tumor growth (7), but the mechanism that underlies overexpression of VEGF-C in cancers remains unclear.

The epigenome, which controls the differential expression of genes in specific cells, is composed of DNA methylation and modifications that occur in DNA-associated components such as histones. It is known that changes in methylation patterns are correlated with the development and progression of tumors (8-10). Methylation of DNA sequences involved in gene regulation (CpG sites) is common in human tumors, including gastric cancer (11). Recent studies have shown that DNA hypomethylation can activate oncogenes and promote tumor progression. For example, *MAGE* (12) and hypomethylation regulation of *synuclein* expression (13) is related to gastric cancer and lymph node metastasis. Transcription of the urokinase gene (*uPA*) induced by 5-aza-CdR, a specific inhibitor of DNA methylation, can

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increase the invasive and metastatic potentials of non-metastatic breast cancer cells (14). The abnormal expression of *uPA* as a result of promoter sequence hypomethylation can also promote the evolution of human prostate cancer (15). These studies clearly show that some of the genes that are activated by hypomethylation are involved in the development of tumors. Understanding the mechanisms underlying these epigenetic changes would provide important information for cancer diagnosis and therapy.

S-adenosylmethionine (SAM) is a biomolecule that is synthesized in all mammalian cells (16) from methionine and ATP by methionine adenosyltransferase. It is a methyl donor in methylation reactions. Acting as a methyl donor, SAM directly affects the degree of DNA methylation and is positively correlated with DNA methylation level. DNA methylation is directly affected by the intracellular concentration of SAM (17). In a recent study, S-adenosylmethionine was used as a promethylation reagent developed for a targeted application (18). SAM promotes apoptosis of tumor cells, but apoptosis activity has not been observed in normal cells (19,20) and it may have potential as a therapeutic reagent for cancer treatments (21,22).

In this study, we investigated how DNA methylation of promoter regions affects gene expression in cancer cells. We found that VEGF-C was hypomethylated in human gastric cancer cells, and that SAM treatment increased its methylation level, thus suppressing gene expression. The suppression of VEGF-C expression was accompanied by inhibition of tumor growth *in vitro* and *in vivo*. Thus, we propose that DNA methylation might be a key factor in expression of VEGF-C, and that SAM might have potential as a chemopreventive agent in gastric cancer.

Material and Methods

Human gastric cancer cell lines

Human gastric cancer cell lines MGC-803, BGC-823, SGC-7901 were purchased from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences. The cells were grown in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA), 1.5 mL glutamine, 2.2 g/L $NaHCO_3$, 100 U/mL penicillin, and 100 U/mL streptomycin, and incubated at 37°C in a humidified atmosphere with 5% CO_2.

Tumor growth in nude mice

Six-week-old male BALB/c mice were from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences and were housed in a specific pathogen-free environment. The Gansu Provincial Hospital Animal Care and Ethics Committee approved the protocol. Mice were randomly separated into three groups of 18 each: MGC-803, BGC-823, SGC-7901. Cells, with a viability of >95%, were suspended in phosphate buffered saline (PBS, 1×10^6 cells in 0.2 mL) and injected into the mice in each group. MGC-803 was injected subcutaneously into the right hind limb; BGC-823 and SGC-7901 were injected subserously into the lesser curvature of the stomach. Seven days later, we selected MGC-803 mice with subcutaneous tumors larger than 60 mm^3, and SGC-7901 and BGC-823 mice with palpable abdominal tumors. Each group was randomly divided into three groups: a control group, a low-dose group, and a high-dose group. SAM was injected into the low-dose (192 $\mu mol \cdot kg^{-1} \cdot day^{-1}$) and high-dose (768 $\mu mol \cdot kg^{-1} \cdot day^{-1}$) groups intraperitoneally daily for 15 days; mice in the control group were given normal saline. The 54 mice were examined once every 4 days, animals were sacrificed after 16 days, and tumor volumes were calculated as vol (mm^3) = 1/2 × (width in $mm)^2$ × length in mm. All animal studies were approved by the Gansu Provincial Hospital Ethics Committee.

Real-time quantitative PCR (RT-qPCR)

An amount of 100 ng of total RNA was prepared from cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Primers designed for VEGF-C were 5′-CAAGGTCGGGCAGGAAGAG-3′ as forward and 5′-TAGAAGGCACAGTCGAGG-3′ as reverse, giving a product total of 231 bp. *GAPDH* was applied as the internal control and amplified with the following primers: 5′-TTCGACAGTCAGCCGCATCTT-3′, 5′-ATCCGTTGACTCCGACCTTCA-3′, with a 90 bp product. The PCR cycling and the detection conditions were as follows: denaturing at 95°C for 2 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 20 s. Estimates of the VEGF-C transcript levels were quantified by the comparative CT method. The relative expression of VEGF-C was calculated as follows: ΔCt(target gene) = Ct(target gene)–Ct (GAPDH). ΔΔCt = ΔCt(target gene)–ΔCt(standard) mean of target gene. The relative copies of the target gene were determined as $2^{-\Delta\Delta Ct}$.

Western blotting

Cultured cells were lysed in protein extraction reagent. The lysates containing 50 μg protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 70 min and then transferred onto polyvinylidene difluoride sandwich membranes (Millipore, USA). The membrane was incubated with rabbit anti-VEGF-C antibody (1:200 dilution, Life Technologies, USA) overnight at 4°C. After incubation with the pig anti-rabbit antibody coupled to horseradish peroxidase (dilution 1:2,000; Santa Cruz, USA), immunoreactive bands were visualized with an enhanced chemilumescent reagent (Amersham Biosciences, USA).

Immunohistochemistry

The xenogafts taken from the nude mice were embedded in paraffin blocks and sectioned (4 μm) for immunostaining. Xylene was used to dewax sections (2 × 5 min) and hydrated in a graded ethanol series (100% down to 75%). The sections were incubated in 3% H_2O_2

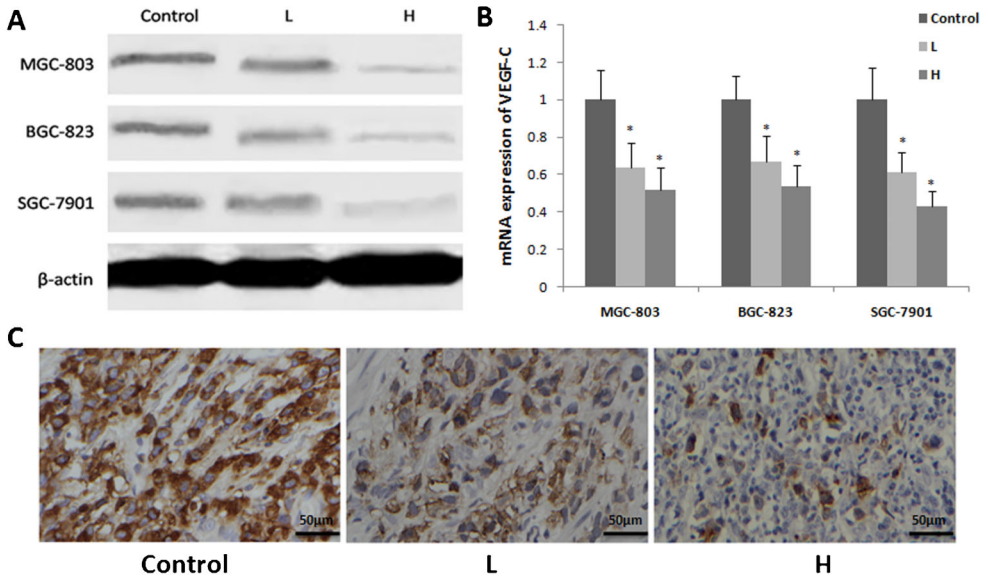

Figure 1. Vascular endothelial growth factor-C (VEGF-C) expression was downregulated after S-adenosylmethionine (SAM) treatment. *A*, Western blot analysis of VEGF-C protein level in gastric cancer cells. L: 2 mM SAM; H: 4 mM SAM. *B*, RT-qPCR analysis of VEGF-C mRNA level in gastric cancer cells. *P<0.05 *vs* control. L: 2 mM SAM; H: 4 mM SAM. *C*, Immunohistochemical analysis of VEGF-C expression in MGC-803 tumor xenografts. L: 192 μmol·kg^{-1}·day^{-1} SAM; H: 768 μmol·kg^{-1}·day^{-1} SAM.

for 10 min and heated at 95°C in citrate buffer, pH 6.0. Then, the sections were blocked with 10% goat serum for 20 min, coated with anti-VEGF-C antibody solution, and incubated at 4°C overnight. On the second day, the tissue slides were washed with PBS three times for 5 min each and then incubated with a secondary antibody at 37°C for 20 min. The sections were then washed with PBS and counterstained with hematoxylin. Images were captured using an XSP-8C microscope (Qianke, China). Each section was examined by two independent observers.

Flow cytometry analysis

Cancer cells were resuspended at 1×10^6 cells/mL and cultured in RPMI 1640 medium for 24 h. Adherent cells were collected by 0.25% trypsinization and washed with PBS. Each sample was resuspended in propidium iodide (PI) stain buffer (2 μL Annexin V-EGFP, 5 μL PI) for 30 min. After treatment, a FACScan (Bipec Biopharma, USA) was used for flow cytometry.

Bisulfite treatment of DNA and methylation analysis

Genomic DNA was extracted with a Genomic DNA Purification Kit (Promega, USA). To examine DNA methylation patterns, genomic DNA was treated with 3 M sodium bisulfite. For analysis of DNA methylation of VEGF-C, we performed bisulfite genomic DNA sequencing analysis. The primers were 5′-GAATATYGYGGGGTGTT TTGGT-3′ (VEGF-C forward) and 5′-ATCCRCTAACRAA AACAAAAATAAAAAC-3′ (VEGF-C reverse); 350 bp product. VEGF-C had 38 CpG sites in the sequence 29, 34, 55, 58, 60, 65, 67, 70, 73, 83, 89, 99, 101, 108, 114, 123, 131, 161, 164, 174, 192, 211, 213, 221, 227, 233, 236, 242, 247, 261, 264, 275, 300, 302, 308, 316, 320, 322, numbered 1-38. Two-microliter aliquots were used as templates for the PCR reactions. Each target sequence was amplified in a 50-μL reaction containing 0.2 μM dNTPs, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.3 μM each primer and 0.75 U of AmpliTaq Gold (Applied Biosystems, USA). PCR amplification consisted of 35 cycles after the initial AmpliTaq Gold activation step. PCR products were purified and cloned into pCR2.1 (Invitrogen). The cloned PCR fragments obtained from each sample were sequenced with M13 forward primer and a PRISM AmpliTaq DNA Polymerase FS Ready Reaction Dye Terminator Sequencing Kit (Applied Biosystems). Reamplified DNA fragments were purified with Centri-Sep Columns (Applied Biosystems) and sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Statistical analysis

All statistical analyses were performed with the SPSS software version 16.0 (SPSS, USA). Data are reported as means ± SD. Statistical analysis was carried out using ANOVA for comparison among multiple groups. In all tests, a P value <0.05 was considered to be statistically significant.

Results

Expression of VEGF-C before and after SAM treatment

To examine the expression of VEGF-C, we performed

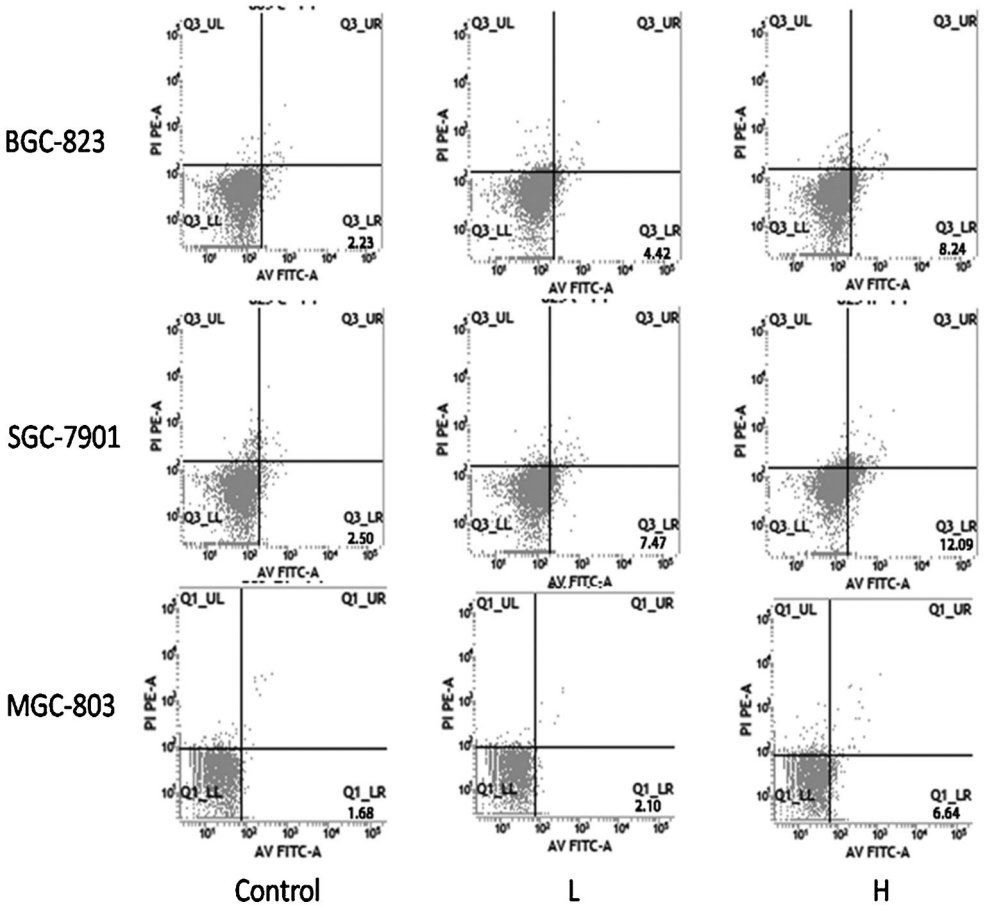

Figure 2. S-adenosylmethionine (SAM) enhanced apoptosis in gastric cancer cells. Flow cytometry analyses of MGC-803, SGC-7901, and BGC-823 after SAM treatment at 2 mM (L) and 4 mM (H) for 3 days compared to negative controls.

Western blotting and RT-qPCR with MGC-803, BGC-823, and SGC-7901 cells. Positive expression of VEGF-C protein was found in all three gastric cancer cell lines (Figure 1A, control). We hypothesized that the expression of VEGF-C protein might be caused by DNA demethylation. To test this hypothesis, MGC-803, BGC-823, and SGC-7901 cells were treated with 2 and 4 mM SAM, and then subjected to Western blotting analysis. VEGF-C protein expression was downregulated in all three gastric cancer cells after SAM treatment (Figure 1A). A similar decrease in VEGF-C mRNA expression was noted in all three gastric cancer cells after SAM treatment compared with the control group (Figure 1B, $P<0.05$).

Similarly, we detected an obvious change in VEGF-C expression in tumor xenografts of MGC-803. After SAM treatment at 192 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ (L) or 768 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ (H) for 15 days, the mice were killed and VEGF-C protein in tumor xenografts was examined by immunohistochemistry. As Figure 1C shows, the expression of VEGF-C protein was effectively suppressed by SAM treatment. These results suggest that DNA methylation may have suppressed VEGF-C expression.

Table 1. Effect of S-adenosylmethionine (SAM) on cell apoptosis *in vitro*.

Group	BGC-823	MGC-803	SGC-7901
Control	2.17 ± 0.14	1.69 ± 0.11	2.49 ± 0.16
SAM (2 mM)	4.43 ± 0.07*	2.12 ± 0.12*	7.43 ± 0.10*
SAM (4 mM)	8.26 ± 0.09*	6.63 ± 0.07*	12.07 ± 0.14*

* $P<0.05$, compared to control (Dunnett *t*-test).

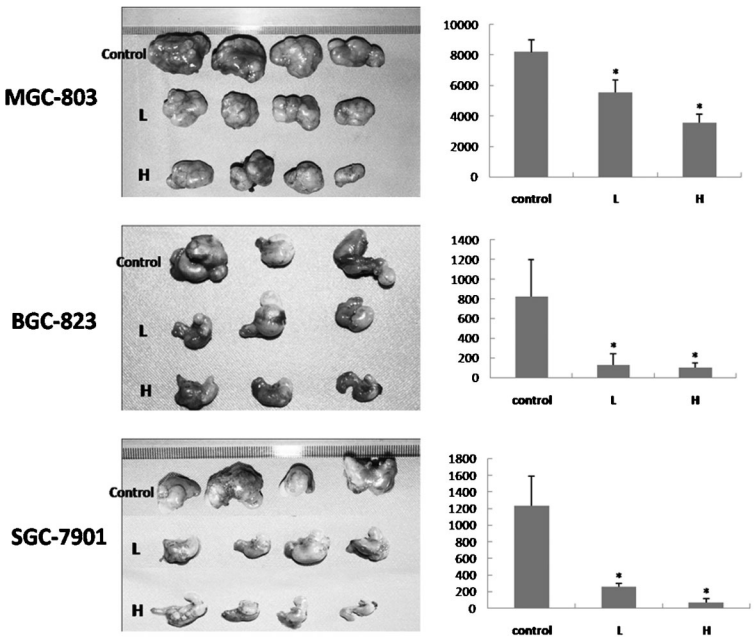

Figure 3. Effect of S-adenosylmethionine (SAM) treatment on tumor growth in nude mice. There was a significant reduction in tumor volume (in mm^3) between SAM treatment group (L: 192 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ SAM, H: 768 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ SAM) and control in MGC-803, SGC-7901, and BGC-823. *$P<0.05$, compared to control.

Methylation status of VEGF-C before and after SAM treatment

To test whether VEGF-C was epigenetically regulated, we performed bisulfite genomic DNA sequencing analysis of MGC-803, BGC-823, and SGC-7901 cells. After cloning the PCR product into a sequencing vector, we randomly selected five colonies for DNA sequencing. As Supplementary Table S1 shows, the VEGF-C promoter present in MGC-803, BGC-823, and SGC-7901 cells was nearly unmethylated, and the percentage of methylated CpG sites was almost 0%. After SAM treatment at 2 mM (L) and 4 mM (H), VEGF-C promoter was highly methylated in the three kinds of gastric cancer cells (Supplementary Table S2). The percentage of methylated CpG sites in MGC-803 was 16.32% (L) and 36.32% (H), 24.74% (L) and 28.42% (H) in BGC-823, and 16.84% (L) and 34.21% (H) in SGC-7901 cells. Meanwhile, VEGF-C was obviously downregulated as described above. This result indicated that SAM was effective for methylation of the VEGF-C promoter present in MGC-803, BGC-823, SGC-7901 gastric cancer cells.

Effect of SAM on cell apoptosis *in vitro*

We investigated apoptosis in MGC-803, BGC-823, and SGC-7901 gastric cancer cells, treated with SAM for 3 days. As shown in Figure 2 and Table 1, flow cytometry showed that SAM increased the amount of apoptotic cells from 2.17 ± 0.14 (control) to 4.43 ± 0.07 (low SAM concentration) and 8.26 ± 0.09 (high SAM concentration) in BGC-823 cells ($P<0.05$). Meanwhile, early apoptosis was greatly enhanced after SAM treatment in both MGC-803 and SGC-7901 cells ($P<0.05$). These results suggested that SAM could promote apoptosis in gastric cancer cells *in vitro*.

Effect of SAM on tumor growth in nude mice

After finding that SAM promoted apoptosis of gastric cancer cells *in vitro*, we evaluated the effect of SAM on tumor growth *in vivo*. After SAM treatment at 192 and 768 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ for 15 days, the mice were killed and the tumor volumes were measured. As shown in Figure 3 and Table 2, ANOVA revealed that the inhibitory effect exerted by SAM treatment at both low and high concentrations on the MGC-803, SGC-7901, and BGC-823 xenografts

Table 2. Effect of S-adenosylmethionine (SAM) treatment on tumor volume in nude mice.

Groups	MGC-803	BGC-823	SGC-7901
Control	8176.1 ± 1492.88	819.95 ± 675.95	1234.20 ± 654.58
SAM (192 $\mu mol \cdot kg^{-1} \cdot day^{-1}$)	5531.8 ± 1701.32*	129.19 ± 212.45*	259.26 ± 64.19*
SAM (768 $\mu mol \cdot kg^{-1} \cdot day^{-1}$)	3526.3 ± 1056.26*	102.20 ± 94.25*	68.395 ± 91.54*

* $P<0.05$, compared to control (Dunnett *t*-test).

was significant compared with controls ($P<0.05$). No toxicity was observed in the mouse experiments, as assessed by changes in behavior, appearance, or weight. These results demonstrated that SAM inhibited tumor growth in nude mice.

Discussion

Epigenetics is the study of changes in gene expression and other phenotypes caused by DNA methylation and histone modification rather than changes in DNA sequence. DNA methylation plays a critical role in regulating and reprogramming gene expression patterns in mammalian cells (23-25). Recent studies have attempted to reverse the hypermethylation of tumor-suppressor genes with DNA demethylation agents (26-28). Emerging data suggest that hypomethylation is involved in cancer metastasis, and invasion by activating particular genes (29,30). DNA hypomethylation may also cause genomic instability and increase the frequency of transposon insertion mutations (31,32). The available data thus supports the hypothesis that DNA hypomethylation controls the activation of oncogenes and it provides valuable insight into developing novel therapeutic strategies against this common disease that target the demethylation machinery. In this study, we found that the VEGF-C promoter present in MGC-803, BGC-823, and SGC-7901 cells was nearly unmethylated, which might be associated with the overexpression of VEGF-C in gastric cancer (33). After treatment by SAM, a methyl donor in numerous methylation reactions, the VEGF-C promoter in the gastric cancer cells was highly methylated and VEGF-C was significantly downregulated.

The overexpression of VEGF-C in primary tumors correlates with poor prognosis, and the prognostic value of VEGF-C overexpression in gastric cancer has been reported (34-36). Therefore, suppression of VEGF-C expression might be an efficacious therapeutic strategy. Recent studies have found that VEGF-C expression was suppressed by lentivirus-mediated VEGF-C siRNA, which inhibited growth of primary breast cancer tumors in the MDA-MB-231 cell xenograft model (37). Other studies have shown that after transfection of *in vitro*-cultured SGC-7901 gastric cancer cells with recombinant pCI-neo-anti VEGF-C plasmids, expression of VEGF-C was reduced and cell proliferation was inhibited (38). Our previous studies demonstrated that VEGF-C-shRNA can successfully inhibit VEGF-C expression in gastric cancer cells and has an effect on primary tumor growth (39). In this study, after showing that SAM suppressed the expression of VEGF-C, we also investigated its effect on tumor growth. Both a proapoptotic effect in gastric cancer cells and tumor growth inhibition in nude mice were observed. Owing to the adverse role of VEGF-C in gastric cancer, we thought that inhibition of VEGF-C expression might play an important role in the antitumor effect of SAM.

SAM acts as an inhibitor of intracellular demethylase activity, which results in hypermethylation of DNA and it is essential as the principal biological methyl donor and precursor for polyamines in most cells (16). Currently, SAM is often used in studies of oncogene inhibition. SAM can effectively inhibit the growth of tumor cells by reversing DNA hypomethylation of promoters of oncogenes such as c-myc and H-ras, thus downregulating their expression (40). In this study, we found that SAM was able to effectively induce VEGF-C methylation and downregulate VEGF-C expression. Our data also show that SAM significantly inhibited tumor growth *in vitro* and *in vivo*. We propose that SAM, as a DNA hypermethylating agent, could be used as a novel therapeutic drug to silence oncogenes and block the progression of gastric cancer.

We conclude that DNA methylation regulates expression of VEGF-C and that SAM can effectively induce VEGF-C methylation, reduce the expression of VEGF-C, and inhibit tumor growth. DNA methylation may be a key factor in the expression of oncogenes such as VEGF-C, and in tumor growth.

Supplementary Material

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Acknowledgments

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