Aberrant methylation of the \textit{SPARC} gene promoter and its clinical implication in gastric cancer

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Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or BM-40, is a multifaceted secreted glycoprotein which is expressed in many types of cells and is associated with tissue remodeling, wound repair, morphogenesis, cellular differentiation, cell migration and angiogenesis. SPARC is differentially expressed in tumors and its surrounding stroma in various cancers. Higher levels of SPARC expression have been reported in breast cancer, melanomas, and glioblastomas. Lower levels of SPARC expression have been found in other types of cancers, such as ovarian, colorectal, and pancreatic cancers, and acute myelogenous leukemia.

SPARC may promote vascularization of tumors, tumor progression and/or invasiveness by modulating the activity of cytokines and stimulating secretion of tissue remodeling metalloproteases. Owing to the correlation between expression and invasion, SPARC was thought to be a proinvasive protein. However, SPARC was found to be significantly downregulated in ovarian cancer cells, and restoring its expression led to decreased tumor growth and apoptosis. Our laboratory has previously shown that SPARC is a tumor suppressor gene, and it appears to mediate, through its suppressive effects on MMP-7 and VEGF, inhibition of gastric cancer growth.

The purpose of our study was to investigate the mechanism by which gastric carcinoma cells downregulate SPARC expression. We hypothesized that epigenetic silencing of SPARC gene by aberrant methylation during gastric carcinogenesis was responsible for the downregulation of SPARC. Here, we examined mRNA and protein expression, and methylation of SPARC in gastric cancer cell lines, and examined the methylation and protein expression in primary tumors. We also correlated these findings with clinicopathological features.
Methods

Ethics statement. The Medical Ethics Committee of Peking University First Hospital approved this clinical study. The experiment was performed in accordance with approved guidelines. Informed written consent was obtained from the patients or their guardians and healthy control subjects.

Cell culture and tumor tissue samples. Human gastric cancer cell lines, BGC-823, MGC-803, SGC-7901 and three control gastric epithelial cell lines (GES-1)16, MKN-4517, KATO III, SUN-1, SUN-16, AGS, NCI-N87(ATCC), were grown in RPMI-1640 medium (Life Technologies Inc., Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and incubated in 5% CO2 at 37 °C. From 2003–2007, a total of 220 surgically resected samples were obtained from patients with gastric cancer who had not received treatment prior to resection at the Peking University First Hospital, P.R. China. Samples were immediately frozen and stored at −80 °C until use.

Reverse transcription-polymerase chain reaction (RT-PCR) assay. A RT-PCR assay was used to determine SPARC mRNA expression. Total RNA was extracted from cultured cells with Trizol (Life Technologies, Rockville, MD, USA) following the manufacturer’s instructions. RNA was reverse transcribed using AMV reverse transcriptase (A3500, Promega, Madison, WI, USA), and aliquots of the reaction mixture were used for subsequent PCR amplification.

Primers for SPARC amplification were as follows: forward primer, 5′-GTTGGG-CAAAAAGGATTACAAACA-3′; and reverse primer, 5′-GGAGGGGGTTGGAAGGAGG-3′. The expected product size of the SPARC cDNA was 512 bp. PCR amplifications were performed in 25 μl reaction volumes containing 0.2 mM dNTPs, 20 pmol of each oligonucleotide primer and 0.2 μl Taq polymerase in PCR buffer. PCR amplification was performed using a PCR Thermal Cycler with an initial denaturation at 95 °C for 5 min, followed by 25 cycles of 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. The housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to confirm the success of RT reaction. Primers for GAPDH amplification were as follows: forward primer, 5′-CACGTTCTTCACCCAAGCTATG-3′; and reverse primer, 5′-GCTTCCACCCACCTTCTTTGATGTCA-3′. PCR amplification was carried out with an initial denaturation at 95 °C for 5 min, followed by 25 cycles of 94°C for 30 s, 65°C for 45 s, and 72°C for 30 s. PCR products were analyzed on 2% agarose gel and visualized using ethidium bromide staining.

Western blotting. Protein lysates from cultured cells, tumors, and normal gastric tissue were extracted by RIPA buffer (Cell Signal Technology) containing protease inhibitors (Cell Signal Technology). Cells were washed in cold Dulbecco’s phosphate-buffered saline (PBS, Sigma), lysed in RIPA buffer containing sodium orthovanadate and a cocktail of protease inhibitors, and sonicated as previously described18. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Lysates were mixed with 1× SDS sample buffer, boiled for 5 min and analyzed using SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane and blocked with 5% BSA in PBS. Membranes were washed with PBS containing 0.2% Tween 20 (PBS-T) and incubated with a rabbit anti-human SPARC monoclonal antibody (Cell Signal Technology; 1: 1000) overnight. After washing with PBS-T, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signal Technology) for 1 h. Signals were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Millipore). Measurements were performed using a Kodak image Station 4000 mm Pro System (Kodak, Rochester, NY, USA).

DNA extraction and sodium bisulfite conversion. DNA was obtained from gastric cancer cell lines, gastric tumor tissue sections (n=220) and normal gastric mucosa samples (n=40). DNA from peripheral blood lymphocytes of gastric cancer patients (n=10) was also extracted. DNA from peripheral blood lymphocytes of healthy nonsmoking volunteers (n=20) was used as a negative control for methylation specific assays. QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer’s recommended protocol. An EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) was used according to the manufacturer’s instructions for sodium bisulfite treatment of genomic DNA. The bisulfite converted DNA was resuspended in 15 μl of elution buffer and stored at −20°C until use.

Methylation-specific polymerase chain reaction (MSP) and DNA sequencing. Methylation status of SPARC gene was determined using MSP as described previously19. Bisulfite-treated DNA (1 μg) was amplified using primers specific for either the methylated or the unmethylated DNA under the following conditions: 95 °C for 5 min; 8 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; and a final extension for 10 min at 72 °C. To detect the 5′-TTTATTAGTGTTTGGAGAGTG-3′ (forward) and 5′-AATCAACAAATATACAAAATAATCATC-3′ (reverse) for unmethylated reactions (132 bp), and 5′-GAGGCGCGTTTGGTTGTGTC-3′ (forward) and 5′-AAGGCGTAAAAAGAAAATAGTCG-3′ (reverse) for methylated reactions (112 bp)20. PCR product (8 μl) was loaded onto a 2% agarose gel and visualized using ethidium bromide staining. PCR products were then subjected to direct sequencing.

5-Aza-2′-deoxycytidine (5-Aza-CdR) treatment. Tumor cell lines with SPARC hypermethylation and absent gene expression were incubated in culture medium with 5 μM 5-Aza-CdR for 6 days, with medium changes at days 1, 3 and 5. Cells were harvested for RNA, DNA and protein on day 6, as described above.

Immunohistochemistry. Immunohistochemistry was performed as previously described21. Sections (4 μm) were cut onto coated slides and deparaffinized using routine techniques. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0), heated at 95 °C in a steamer for 20 min. After blocking endogenous peroxidase activity with a 3% aqueous H2O2 solution for 5 min, sections were incubated with an anti-SPARC monoclonal antibody at a final concentration of 4 μg/ml for 60 min. Immunolabeled signals were detected with the EnVision Plus Detection Kit (DAKO, Carpinteria, CA, USA) following the manufacturer’s protocol. Sections were counter-stained with hematoxylin. The extent of immunolabeling of SPARC was scored as follows: 0%, negative; ≤10%, focal; and >10%, positive. The intensity of immunolabeling was scored as weak (+), moderate (+ +) or strong (+ + +).

Cell proliferation, invasion and migration assay. To investigate the cell proliferation, four cell lines (BGC-823, SUN-1, MGC-803, HGC-27) exposed to 5 μM 5-Aza-CdR for 3 days were seeded at 10,000 cells per well in a 48-well plate. Cells were counted from duplicate wells at 24, 48 and 72 h. Results were based on three independent experiments. The cells without 5-Aza-CdR treatment were assayed at the same time as the controls.

Invasion assay was performed in a six-well Transwell chamber (Costar Corporation, Tewksbury, MA, USA) that contains an 8 μm pore size polycarbonate membrane precoated with 30 μg/ml Matrigel (BD Biosciences, Bedford, MA, USA). Cells were re-suspended in a serum-free medium at a concentration of 5×104 cells/ml, and seeded in the upper compartment of chamber and incubated in the presence of 5-Aza-CdR for 24 h. A medium containing 10% fetal calf serum was added to the bottom chamber. After reculturing with 5% CO2 at 37 °C for 24 hours, the Transwell chambers were inverted and stained with hematoxylin. The migration assay was done in a similar manner, but without Matrigel coated on the filters. The invasion and migration assays were done in triplicate. Five fields were randomly selected for cell counting on the membranes.

Statistical analysis. Continuous variables were shown as the mean ± SD, and differences between groups were evaluated using unpaired Student’s t-test. The relationship between SPARC expression and clinicopathological parameters were analyzed using Fisher’s exact test, chi-square test, or Pearson χ2 and Mann-Whitney U test if necessary. Survival was calculated from the date of initial diagnosis until death or the date of the last follow-up. Survival was analyzed according to the Kaplan-Meier method, and differences in their distribution were evaluated by means of the log-rank test. A multivariate Cox proportional hazards model was developed to evaluate the joint effects of covariates. P values were two-sided, and a P value of less than 0.05 was defined as being statistically significant. Statistical analyses were conducted using the IBM SPSS Statistics software package (version 20, IBM-SPSS Statistics, Armonk, NY, USA).

Results

Aberrent methylation and expression of SPARC gene in cell lines. Expression of SPARC in cell lines was examined using RT-PCR, and the representative data are shown in the upper bands of Figure 1 A. Loss of SPARC expression was observed in 7 of 10 (70%) gastric cancer cell lines, while aberrant methylation was found in 8 of 10 (80%) of them (Figure 1 B). Concordance between loss of gene expression and aberrant methylation of SPARC was 70% (7 of 10) in gastric cancer cell lines. Both SPARC mRNA expression and aberrant methylation were detected in SGC-7901 cell lines.

Western blotting. Western blotting showed that SPARC was undetectable in AGS, MKN-45, NCI-N87, BGC-833, KATO III, SUN-1 and SUN-16 cell lines, but detectable in GES-1, HGC-27, SGC-7901 and MGC-803 cell lines (lower bands of Figure 1 A).

5-Aza-2′-deoxycytidine (5-Aza-CdR) treatment. To confirm that methylation of SPARC gene was responsible for the loss of SPARC protein expression, gastric cancer cell lines were treated with the demethylating agent 5-Aza-CdR. 5-Aza-CdR treatment was able to restore SPARC mRNA expression in cell lines (BGC-833, AGS, NCI-N87, MKN-45, KATO III, SUN-1, SUN-16) that did not constitutionally express SPARC (upper bands, Figure 1 C). Moreover, protein expression was restored in the seven cell lines (BGC-833, AGS, NCI-N87, MKN-45, KATO III, SUN-1, SUN-16) previously lacking SPARC expression (lower bands, Figure 1 C).
Methylation analysis of SPARC gene in gastric cancer cell lines. Bisulfite treated genomic DNA samples from SGC-7901, BGC-823, AGS, NCI-N87, MKN-45, KATO III, SUN-1 and SUN-16 cell lines were amplified using methylated primers, and the PCR products were separated and visualized in 2% agarose gels. M = methylated band; U = unmethylated band. The results showed that all of the CpG sites in this region were methylated in the 8 gastric cancer cell lines (Figure 2).

Immunostaining of SPARC protein in gastric cancer tissues. SPARC protein expression was examined in 172 primary gastric carcinoma tissues and 10 normal gastric tissues using immunohistochemistry and an anti-SPARC monoclonal antibody. In 134 (78%) of 172 cases, moderate (+ +) to strong (+ + +) SPARC expression was found in stromal cells, presumably fibroblasts (Figure 3). SPARC expression was observed in neoplastic epithelium in 58 (34%) of 172 cases, but 15 of 58 cases of the immunostaining was weak and focal. In the remaining 114 cases (66%), neoplastic cells did not express SPARC throughout the tumor. Of 172 cases, 116 cases showed negative (0) or weak (+) expression of SPARC protein as well as methylation of SPARC gene, and 43 cases showed moderate (+ +) to very strong (+ + +) expression without methylation of the gene (Figure 4E).

Aberrant methylation of SPARC gene in primary tumors. Results of SPARC methylation in primary tumors (n = 220), normal gastric mucosa samples (n = 40), peripheral blood lymphocytes from patients (n = 10) and healthy nonsmoking volunteers (n = 20) are detailed in Table 1 and Figure 4. SPARC methylation was a tumor-specific event in gastric cancers (P < 0.001) as compared with the corresponding adjacent non-malignant tissues. Of the 220 gastric cancers, methylation occurred in 163 (74%) samples. When comparison was made between SPARC methylation and clinicopathological data, we found that SPARC methylation was unrelated to gender and age. Remarkably, the overall survival was poorer in intestinal gastric cancer patients with...
SPARC methylation than in those without methylation (P < 0.001, log-rank test; Figure 5A). The relationship between overall survival and SPARC methylation in cancers was also found in the subgroups of patients at different stages (P = 0.002 for those at stages I and II; P < 0.001 for those at stages III and IV; Figure 5B and 5C), different tumor sizes (P = 0.03 for those with tumor size < 2.5 cm; P < 0.001 for those ≥ 2.5 cm; Figure 5D and 5E), and lymph node metastasis (P = 0.001 for those without lymph node metastasis; P < 0.001 for those with lymph node metastasis; Figure 5F and 5G). Using the multivariate Cox proportional hazards model, we found that SPARC methylation was an independent adverse prognostic factor (P < 0.001; RR 2.754, 95% CI 1.780–4.261), similar to the clinically well-known prognostic factors of disease stage (P = 0.002; RR 2.334, 95% CI 1.351–4.034), tumor size (P = 0.012; RR 3.784, 95% CI 1.332–10.747) and lymph node metastasis (P = 0.020; RR 1.824, 95% CI 1.100–3.024) in adenocarcinoma cases (Table 2).

Cell proliferation, invasion and migration assay. Treatment of 5-Aza-Cdr induced the expression of SPARC in gastric cancer cell lines (Figure 1C). We then examined the changes of cell proliferation, invasion and migration of the 4 gastric cancer cell lines (BGC-823, SUN-1, MGC-803 and HGC-27) after incubation with 5-Aza-Cdr. In BGC-823 and SUN-1 cell lines that had SPARC methylation (Figure 1B), cell proliferation decreased significantly after 5-Aza-Cdr treatment, beginning as early as 24 h of the treatment (Figure 6A). In contrast in MGC-803 and HGC-27 cell lines that had unmethylated SPARC and expressed SPARC (Figure 1A and 1B), decrease of cell proliferation was less prominent after 5-Aza-Cdr treatment, and was only noticeable after 2 days (Figure 6A). Similarly, cell migration (Figure 6B) and invasion (Figure 6C) were also decreased more in BGC-823 and SUN-1 cell lines and less in MGC-803 and HGC-27 cell lines after 5-Aza-Cdr treatment.

Discussion
In this study, we attempted to investigate the mechanism through which SPARC expression was reduced in human gastric carcinomas. Our results showed that the downregulation of SPARC was resulted from the hypermethylation of promoter region in SPARC gene. Previous studies from several groups have indicated methylation in SPARC promoter region resulting in the downregulation of SPARC expression in multiple neoplasms, including colon, pancreatic, ovarian and endometrial carcinomas. In this study, SPARC...
Expression was lost in association with the aberrant DNA methylation in SPRAC gene in most gastric cell lines and surgical gastric cancer samples, and the loss of SPARC expression could be rescued in gastric cancer cell lines upon treatment with the demethylating agent 5-Aza-CdR. Methylation of specific CpG sites in SPRAC gene was consistently detected in gastric cancer cell lines by MSP and sequencing. Although other mechanisms may also account for the downregulation of SPARC expression, the relationship between downregulation of SPARC expression and methylation in SPRAC gene was confirmed by our excellent uniformity among mRNA expression by RT-PCR, protein expression by western-blotting, immunostaining, and DNA methylation in SPRAC gene by MSP and sequencing in gastric cancer cell lines and primary tumors. 5-Aza-CdR is a nucleoside anti-metabolite agent and a potent inhibitor of DNA methyltransferase.

Figure 4 | (A) Representative examples of the MSP assay using DNA from primary gastric tumors. PCR products were visualized in a 2% agarose gel stained with ethidium bromide. (B) Representative examples of the MSP assay using DNA from normal gastric mucosa samples. (C) Representative examples of the MSP assay using DNA from peripheral blood lymphocytes from healthy volunteers. (D) Representative examples of the MSP assay using DNA from peripheral blood lymphocytes from patients. M = methylated; U = unmethylated. (E) Four cases of SPARC expression and SPRAC gene methylation in tumors and adjacent normal gastric tissues.

| Table 1 | SPARC methylation in gastric cancer cell lines, primary gastric cancers, and controls |
|---|---|---|
| Samples | Total no. | No. Methylated (%) |
| Gastric cancer cell line | 10 | 8(80) |
| Primary gastric cancer | 220 | 163(74) |
| Intestinal type | 185 | 136(74) |
| Diffuse type | 35 | 27(77) |
| Nonmalignant sample | 70 | 70(100) |
| Normal gastric mucosa | 40 | 40(100) |
| Peripheral blood mononuclear cells* | 20 | 20(100) |
| Peripheral blood mononuclear cells† | 10 | 10(100) |

*From healthy nonsmoking volunteers.  †From patients.
Figure 5 | Kaplan-Meier plots of overall survival in intestinal gastric cancer patients. (A) Survival of patients with \(n = 136\) or without \(n = 49\) SPARC methylation. (B) Survival of patients with early stages I or II, with \(n = 14\) or without SPARC methylation \(n = 23\). (C) Survival of patients with advanced stages III or IV, with \(n = 122\) or without SPARC methylation \(n = 26\). (D) Survival of patients with tumor sizes < 2.5 cm, with \(n = 5\) or without SPARC methylation \(n = 14\). (E) Survival of patients with tumor sizes \(\geq 2.5\) cm, with \(n = 131\) or without SPARC methylation \(n = 35\). (F) Survival of patients lymph node metastasis negative, with \(n = 21\) or without SPARC methylation \(n = 16\). (G) Survival of patients lymph node metastasis positive, with \(n = 115\) or without SPARC methylation \(n = 33\). Probability of survival curves was calculated using the Kaplan-Meier product-limit method and compared via the log-rank test between groups.
of DNA methyltransferase 1 activity. 5-Aza-Cdr does not specifically inhibit the methylation in SPARC gene, but has a global effect on other methylated genes. However, in our findings, 5-Aza-Cdr obviously inhibited the cell proliferation, invasion, and migration in the gastric cell lines with methylation in SPARC gene.

The downregulation of SPARC expression in gastric cancers was also found in pancreatic cancers, which usually exhibited the loss of SPARC and the higher expression level in normal epithelial cells. Upregulation of SPARC was unusually present in stromal cells distant from the pancreatic cancers. These findings indicated that there

| Parameters                          | No. (%) | Methylation (%) | Unmethylation (%) | P-Value | Univariate Risk ratio 95% CI P-value | Multivariate Risk ratio 95% CI P-value |
|-------------------------------------|---------|----------------|-------------------|---------|--------------------------------------|---------------------------------------|
| N = 185                             | N = 136 | N = 49         |                   | P = 0.613 | 1.268 0.892–1.803 P = 0.185           |                                       |
| Gender                              |         |               |                   |         |                                      |                                       |
| Male                                | 100 (54.1) | 72(72.0)   | 28(28.0)          | P = 0.271 | 1.045 0.731–1.494 P = 0.810           |                                       |
| Female                              | 85 (45.9)  | 64(75.3)    | 21(24.7)          | P = 0.002 | 3.784 1.332–10.747 P = 0.012          |                                       |
| Age (years) ≤63`                    | 103 (55.7) | 79(76.7)    | 24(23.3)          | P = 0.007 | 2.334 1.351–4.034 P = 0.002          |                                       |
| <63                                 | 82 (44.3)  | 57(69.5)    | 25(30.5)          | P = 0.010 | 1.824 1.100–3.024 P = 0.020          |                                       |
| Size (cm) ≤2.5                      | 19 (10.3)   | 5(26.3)     | 14(73.7)          | P = 0.001 | 1.824 1.100–3.024 P = 0.020          |                                       |
| ≥2.5                                | 166 (89.7) | 131(78.9)   | 35(21.1)          | P < 0.001 | 2.334 1.351–4.034 P = 0.002          |                                       |
| TNM stage I, II                     | 37 (20.0)   | 14(37.8)    | 23(62.2)          | P < 0.001 | 2.754 1.780–4.261 P < 0.001          |                                       |
| III, IV                             | 148 (80.0) | 122(82.4)   | 26(17.6)          | P < 0.001 | 2.754 1.780–4.261 P < 0.001          |                                       |
| Lymph node metastasis               |          |               |                   | P = 0.010 | 1.824 1.100–3.024 P = 0.020          |                                       |
| Negative                            | 37(20.0)   | 21(56.8)    | 16(43.2)          | P = 0.010 | 1.824 1.100–3.024 P = 0.020          |                                       |
| Positive                            | 148 (80.0) | 115(77.7)   | 33(22.3)          | P = 0.010 | 1.824 1.100–3.024 P = 0.020          |                                       |

Prognostic factors for overall survival was conducted by univariate analyses using the log-rank test and multivariate analyses using the Cox proportional hazards model. CI, confidence interval.

Pearson’s $x^2$ test

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Figure 6 | Changes of cell proliferation, invasion, and migration in BGC-823, SUN-1, MGC-803, HGC-27 cell lines after 5-Aza-Cdr treatment. Decrease of cell proliferation (A), cell migration (B) and cell invasion (C) were more in BGC-823 and SUN-1 cell lines than in MGC-803 and HGC-27 cell lines after 5-Aza-Cdr treatment. *: P < 0.01.
was a complex pattern of simultaneous selected downregulation in a specific cell type (tumor cells) accompanied by selected upregulation in adjacent stromal cells. In our findings, SPARC was expressed in stromal cells and occasionally in tumor cells, similar to the findings in a previous report on gastric cancer study22; the loss of SPARC gene expression was associated with aberrant hypermethylation in SPARC gene and could be reversed by 5-Aza-Cdr treatment23, similar to the evidences previously described in pancreatic, ovarian and breast cancer cells24.25,26.

The phenomenon that hypermethylation of specific CpG sites in SPARC gene in most gastric cancer cell lines may suggest the usefulness of SPARC expression in cells as a diagnostic or predictive marker for gastric cancers. Complete or partial loss of SPARC expression in some stomach epithelia with morphologically normal appearance may in fact represent an early epigenetic event predisposing to become gastric cancer cells. This hypothesis requires prospective studies to be determined, but a similar pattern has been previously described for colorectal cancers24.

In our findings, higher stage (III or IV), larger tumor size (≥2.5 cm) and positive lymph node metastasis were apparently associated with a poor prognosis in gastric cancers as described in another report27. Additionally, patients with methylation in SPARC gene in gastric cancers were associated with a poorer prognosis than those without methylation. SPARC expression was downregulated in lung, pancreatic and ovarian cancers, but was upregulated in metastatic prostate, bladder and hepatocellular cancers. SPARC expression in normal and tumor cells were highly dependent on tumor type and culture conditions. SPARC expression in cancer tissues correlated with poor prognosis in malignant melanoma, bladder and esophageal cancer as reported by others, but some of these reports were solely based on the results of RT-PCR on whole specimens28–30. Therefore, aberrant expression of SPARC in primary tumors may be related to poor prognosis. However, some recent studies reported that SPARC were associated with poorer prognosis of the gastric cancer patients31–34. Our findings, however, demonstrated that patients without SPARC methylation was associated with a good disease outcome and a better long-term survival. Our study also showed that SPARC expression in stromal cells was significantly higher than that in cancer cells, and 5-Aza-Cdr inhibited the cell proliferation, invasion and migration in the gastric cell lines with methylation in SPARC gene. Our laboratory previous research also showed SPARC suppresses angiogenesis of gastric cancer by down-regulating the expression of VEGF and MMP-728. Others considered that this suppression might be related to the tumor growth, and SPARC had an antiproliferative function through modulating cell cycle regulatory proteins or growth factors29. Similar results have been reported in pancreatic cancer30.

Our results suggest the potential of clinical applications. Because that SPARC is frequently methylated in gastric cancers but not in normal gastric mucosa samples, a useful strategy may develop for gastric cancer diagnosis based on the detection of aberrantly methylation in SPARC gene by MSP in samples such as biopsies, serum, and gastric lavage. Several tumor suppressor genes such as p16 have already been shown to be useful for such a purpose31. The methylation of CpG sites in SPARC gene may be used as a specific diagnostic marker in gastric cancer, to which there is currently no perfect markers for the diagnosis of a noninvasive gastric cancer. Methylation in SPARC gene may also be useful to combine other markers such as circulating tumor cells in blood to predict the prognosis of patients, or to establish the epigenetic type of various tumors to evaluate their differences in sensitivity to chemotherapy, metastasis possibilities and/or overall prognosis32.

In summary, we have identified that SPARC was significantly downregulated in gastric cancer cells due to DNA methylation in SPARC gene. The DNA methylation correlated with the prognosis of gastric adenocarcinomas. We also provided evidence that DNA methylation in SPARC gene may play a role in the pathogenesis of gastric cancers. 1. Kim, J. G. et al. Comprehensive DNA methylation and extensive mutation analyses reveal an association between the CpG island methylator phenotype and oncogenic mutations in gastric cancers. Cancer Lett 330, 33–40 (2013). 2. Nomura, T. et al. Influence of HRH2 promoter polymorphism on aberrant DNA methylation of DAPK and CDH1 in the gastric epithelium. BMC Gastroenterol 13, 1 (2013). 3. Tanaka, T. et al. Loss of trefoil factor 1 is regulated by DNA methylation and is an independent predictive factor for poor survival in advanced gastric cancer. Int J Oncol 42, 894–902 (2013). 4. Hibi, K. et al. Methylation of the WNT5A gene is frequently detected in early gastric carcinoma. Hepatogastroenterology 59, 2661–2 (2012). 5. Min, S. Y. et al. Prognostic significance of phthlamindesoxiase 1 (GPD1) down-regulation and correlation with aberrant promoter methylation in human gastric cancer. Anticancer Res 32, 3169–75 (2012). 6. Takamaru, H. et al. Aberrant methylation of RASGRF1 is associated with an epigenetic field defect and increased risk of gastric cancer. Cancer Prev Res (Phila) 5, 1203–12 (2012).

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Author contributions

Z.Y.C., G.W.C. and Y.C.L. wrote the main manuscript text; Z.Y.C., J.L.Z., H.X.Y., P.Y.W., J.Z., W.W., X.W., Y.L.W. and S.W.C. prepared figures 1–6. All authors reviewed the manuscript.

Additional information

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