ORIGINAL ARTICLE

Aberrant methylation of *SPARC* in human hepatocellular carcinoma and its clinical implication

Ye Zhang, Bin Yang, Zhi Du, Tong Bai, Ying-Tang Gao, Yi-Jun Wang, Cheng Lou, Feng-Mei Wang, Yu Bai

Ye Zhang, Zhi Du, Third Central Clinical College of Tianjin Medical University, Tianjin 300170, China
Ye Zhang, Zhi Du, Tong Bai, Yi-Jun Wang, Cheng Lou, Feng-Mei Wang, Yu Bai, The Third Central Hospital of Tianjin, Tianjin 300170, China
Bin Yang, Ying-Tang Gao, Tianjin Key Laboratory of Artificial Cell, Tianjin 300170, China
Correspondence to: Zhi Du, Professor of Medicine, The Third Central Hospital of Tianjin, Tianjin 300170, China. zhi-du@163.com
Telephone: +86-22-84112148 Fax: +86-22-24315132

Abstract

AIM: To investigate the methylation status of secreted protein acidic and rich in cysteine (*SPARC*) in human hepatocellular carcinoma (HCC) and evaluate its clinical implication.

METHODS: The methylation status of *SPARC* was analyzed in one HCC cell line (SMMC-7721) and 60 pairs of HCC and corresponding nontumorous tissues by methylation-specific polymerase chain reaction and bisulfite sequencing. The expression of *SPARC* mRNA and protein were examined by reverse transcription polymerase chain reaction and immunohistochemistry, respectively. The correlations between the methylation status and the gene expression, the clinicopathological parameters, as well as the prognosis after surgery were analyzed.

RESULTS: In the SMMC-7721 cell line, the loss of *SPARC* expression was correlated with the aberrant methylation and could be reactivated by the demethylating agent 5-aza-2'-deoxycytidine. Methylation frequency of *SPARC* in HCC was significantly higher than that in the corresponding nontumorous tissues (45/60 *vs* 7/60, $P < 0.001$), and it was correlated with the pathological classification ($P = 0.019$). The downregulation of the *SPARC* mRNA expression in HCC was correlated with the *SPARC* methylation ($P = 0.040$). The patients with methylated *SPARC* had a poorer overall survival than those without methylated *SPARC* (28.0 mo *vs* 41.0 mo, $P = 0.043$).

CONCLUSION: Aberrant methylation is an important mechanism for *SPARC* inactivation in HCC and *SPARC* methylation may be a promising biomarker for the diagnosis and prognosis of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most

common malignances and the third leading cause of cancer death in the world[1,2]. To date, surgical resection is still considered the most important treatment for patients with resectable HCC[3]. Unfortunately, most patients are at inoperable stages when the tumor is diagnosed[4]. In addition, the high incidence of tumor recurrence after curative resection also leads to poor clinical outcomes[5,6]. Therefore, the development of biomarkers for early diagnosis and accurate prognosis of HCC is valuable for improving patients' survival.

Although the detailed molecular mechanisms of hepatocarcinogenesis remain largely unclear, the accumulating evidences have shown that aberrant methylation of promoter CpG islands causes inactivation of tumor suppressor genes, which is involved in the occurrence and development of HCC[7-10]. Detections of such an aberrant DNA methylation of tumor suppressor genes could be used as a diagnostic or a prognostic marker for HCC.

Secreted protein acidic and rich in cysteine (*SPARC*) is a matricellular glycoprotein involved in some biological processes, including tissue remodeling, angiogenesis, extracellular matrix production and so on[11-13]. It has been reported that *SPARC* has tumor suppressing properties to various cancers, such as ovarian cancer and pancreatic cancer[14-16]. Moreover, *SPARC* is epigenetically silenced through promoter hypermethylation in these cancers, and the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR) can rescue *SPARC* expression[17-20]. The *SPARC* promoter methylation is an important factor in the carcinogenesis of these cancers and may be a promising epigenetic marker for them. However, up to date, there have been few reports about the methylation status in HCC.

In this study, in order to explore the status of *SPARC* methylation in HCC, we examined the methylation and expression of *SPARC* in HCC cell line and tissues. We correlated the methylation status with clinicopathologic features and evaluated whether the methylation of *SPARC* can serve as a potentially diagnostic or prognostic biomarker for HCC.

MATERIALS AND METHODS

Cell line and patient samples

The SMMC-7721 cell line used in this study was obtained from the Shanghai Institute of Cell Biology (Shanghai, China). HCCs and their corresponding nontumorous tissues were obtained from 60 patients who were diagnosed and treated at the Department of Hepatobiliary Surgery, Tianjin Third Central Hospital in China from October 2003 to June 2008. This study protocol was approved by the Clinical Research Ethics Committee of our institution and the informed consent was obtained from each of these patients. After surgical resection, samples were immediately stored in the liquid nitrogen for later analysis. For the gene expression analysis, the hematoxylin-eosin-stained samples from each tumor block were examined microscopically to confirm

the presence of more than 80% tumor cells. The nontumorous samples from each patient were also microscopically confirmed.

Cell culture and 5-Aza-CdR treatment

SMMC-7721 cells were grown in DMEM supplemented with 100 g/L fetal bovine serum and incubated in 37 ℃ and 50 mL/L CO_2. For the 5-Aza-CdR (Sigma, St Louis, MO, United States) treatment, cells were split to 5×10^5 per 75-cm^2 culture bottle and incubated overnight in the growth media. The normal growth media was replaced with the growth media supplemented with 5-Aza-CdR (10 μmol as a final concentration) for 6 d with the media change on day 4. Cells cultured with vehicle alone served as 5-Aza-CdR negative control. After the culture, cells were harvested for the extraction of genomic DNA and total RNA. In order to detect the *SPARC* protein in different groups by immunocytochemical staining, SMMC-7721 cells were also seeded onto 6-well plates containing coverslips to induce cells to spread and adhere to the glass.

DNA extraction and bisulfite treatment

The genomic DNA was extracted from the cell line and tissue samples by digesting with sodium dodecyl sulfate/proteinase K in Tris ethylenediamine tetraacetic acid (TE) buffer followed by a standard phenol/chloroform extraction. The extracted DNA was subjected to the bisulfite treatment as previously described[21-23]. Briefly, 1-2 μg genomic DNA was denatured with 0.3 mol/L NaOH at 37 ℃ for 20 min, and incubated in 3.0 mol/L sodium bisulfite and 10 mmol/L hydroquinone at 55 ℃ for 16 h. The DNA was desalted with a QIAquick gel extraction kit (Qiagen, Valencia, CA, United States) and dissolved in 50 μL of 10 mmol/L TE buffer (pH 8.0). Then, 5.5 μL of 3.0 mol/L NaOH was added and incubated at 37 ℃ for 20 min to desulfonate it. The modified DNA was neutralized with 30 μL of 10 mol/L ammonium acetate, precipitated using 2 volumes of ethanol, and resuspended in 40 μL of 1.0 mmol/L TE buffer (pH 7.6).

Methylation specific polymerase chain reaction and sequencing

Methylation specific polymerase chain reaction (MSP) was performed to examine the methylation status at CpG island of *SPARC* promoter in both SMMC-7721 cells and tissue samples. The primers used in this study for polymerase chain reaction (PCR) are shown in Table 1. A PCR mixture contained 1 × PCR buffer (10 mmol/L Tris, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ and 10 mmol/L β-mercaptoethanol), deoxynucleotide triphosphates (each at 0.2 mmol/L), primers (10 pmol each), bisulfite-modified DNA templates (2 μL) and 1 U of Taq polymerase, and the final volume was 25 μL. The PCR conditions were as follows: 94 ℃ for 2 min; then 40 cycles of 94 ℃ for 30 s, at optimum annealing temperature for 30 s and 72 ℃ for 30 s; and final extension for 5 min at 72 ℃. The normal leukocyte DNA methylated *in vitro* with SssI

Table 1 Primer sequences for polymerase chain reaction

Gene	Primer sequences (forward/reverse 5'-3')	Accession No.	Location to transcription start	Product size (bp)	Annealing temperature (℃)
SPARC methylation	GAGAGCGCGTTTTGTTTGTC	NM_003118.2	+52 to +71	112	54
	AACGACGTAAACGAAAATATCG		+142 to +163		
SPARC unmethylation	TTTTTTAGATTGTTTGGAGAGTG	NM_003118.2	+36 to +58	132	59
	AACTAACAACATAAACAAAAATATC		+143 to +167		
SPARC BS	GATAGAGATAGTTTTGGTTATGGGA	NM_003118.2	-119 to -95	401	55
	CCACCTTCTAAAAAACA ACAAAC		+260 to +282		
SPARC mRNA	CGCATGCGGGACTGGCTCAA	NM_003118.2	+601 to +620	148	60
	GCTCCACGGGG TGGTC TCCT		+729 to +748		
GAPDH mRNA	GGGCATCCTGGGCTACACTGA	NM_002046.3	+915 to +935	143	58
	CAAATTCGTTGTCATACCAGGAAATG		+1032 to +1057		

SPARC: Secreted protein acidic and rich in cysteine; BS: Bisulfite sequencing; *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase.

methyltransferase (New England Biolabs Inc., Beverly, MA, United States) was used as the positive control of methylation, and the normal leukocyte DNA was used as the negative control. The distilled water without template DNA was used as a blank control for all tests. Five microliters of PCR products underwent electrophoresis on 25 g/L agarose gel, and was visualized under ultraviolet illumination with the ethidium bromide staining. To verify the accuracy of MSP, the PCR products of both methylation and unmethylation were randomly chosen and cloned into the pMD-18-T vector (TaKaRa, Dalian, China) followed by a sequencing analysis.

To investigate the status of CpG sites in the region of *SPARC* promoter of SMMC-7721 cells, bisulfite sequencing analysis was performed for the bisulfite-treated DNA. The PCR products were cloned into a pMD-18-T vector and 8 individual clones of each group were sequenced.

RNA preparation and reverse transcription-PCR

RNA was extracted from the cell line and tissues using the Trizol (Tiangen, Beijing, China) according to the manufacturer's instructions. The total mRNA was digested with the DNase I (Ambion, Austin, TX, United States) to remove the genomic DNA contamination and then subjected to reverse transcription using the reverse transcription system (Promega, Madison, WI, United States). *SPARC* expression of SMMC-7721 cells and tissues were tested by reverse transcription (RT)-PCR and quantitative RT-PCR, respectively. Real-time quantitative RT-PCR was done on the ABI Prism 7000 sequence detection system in combination with the SYBR green real-time PCR master mix (Toyobo, Shanghai, China). The PCR amplification was carried out for 2 min at 94 ℃ for the initial denaturation, followed by 35 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 30 s. Melting curve analyses following amplification were performed to assure the product specificity. The relative expression of *SPARC* mRNA was normalized to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) in the same cDNA using the comparative CT method. For the quantification of gene expression, the target gene (*SPARC*) value normalized to the expression of *GAPDH* was designated as ΔCT [ΔCT = CT (*SPARC*) - CT

(*GAPDH*)]. The ΔCT for the nontumorous samples was then subtracted from the ΔCT for the tumorous samples to generate $\Delta\Delta$CT [$\Delta\Delta$CT = ΔCT (tumor) - ΔCT (nontumorous sample)]. The $\Delta\Delta$CT measurement was used to calculate the relative expression ($2^{-\Delta\Delta CT}$).

Immunohistochemistry

The protein expression of *SPARC* was examined in 23 primary HCCs and the corresponding nontumorous tissues by immunohistochemistry. Sections (5 μm) from the tumor and nontumorous tissues were cut onto coated slides and deparaffinized by the routine techniques. The antigen retrieval was performed in 10 mmol sodium citrate buffer (pH 6.0), and heated at 95 ℃ for 10 min. After endogenous peroxidase activity was blocked with 30 g/L H$_2$O$_2$ for 5 min, the sections were incubated with an anti-*SPARC* monoclonal antibody at a 1:100 dilution (Santa Cruz Biotechology, United States) overnight. Labeling was detected with the PV-9000 Kit (Zhongshan, Beijing, China), following the protocol afforded by the manufacturer, and all sections were counterstained with hematoxylin. Cytoplasm staining of more than 90% parenchyma cells (tumor cells or liver cells) was regarded as positive for *SPARC*.

Similarly, *SPARC* protein was also tested in SMMC-7721 cells growing on the coverslips by immunocytochemistry.

Analysis for clinicopathological data and statistics

The gene methylation status in HCC was evaluated in the correlation with the clinicopathological parameters of patients, including age, gender, tumor size, virus infection, liver function, tumor number, vascular infiltration, pathology class and the level of alpha-fetal protein (AFP). The Pearson χ^2 test or the Fisher's exact test was used to analyze associations between methylation frequencies and categorical variables. Disease free or overall survival was calculated from the date of the operation until tumor recurrence or death or the date of the last follow-up (censored). Survival was analysed by the Kaplan-Meier method, and differences in their distribution were evaluated by the log-rank test. A multivariate Cox's proportional-hazard model was developed to evaluate the covariates' joint effects. All *P* values were two-sided, and *P* value less
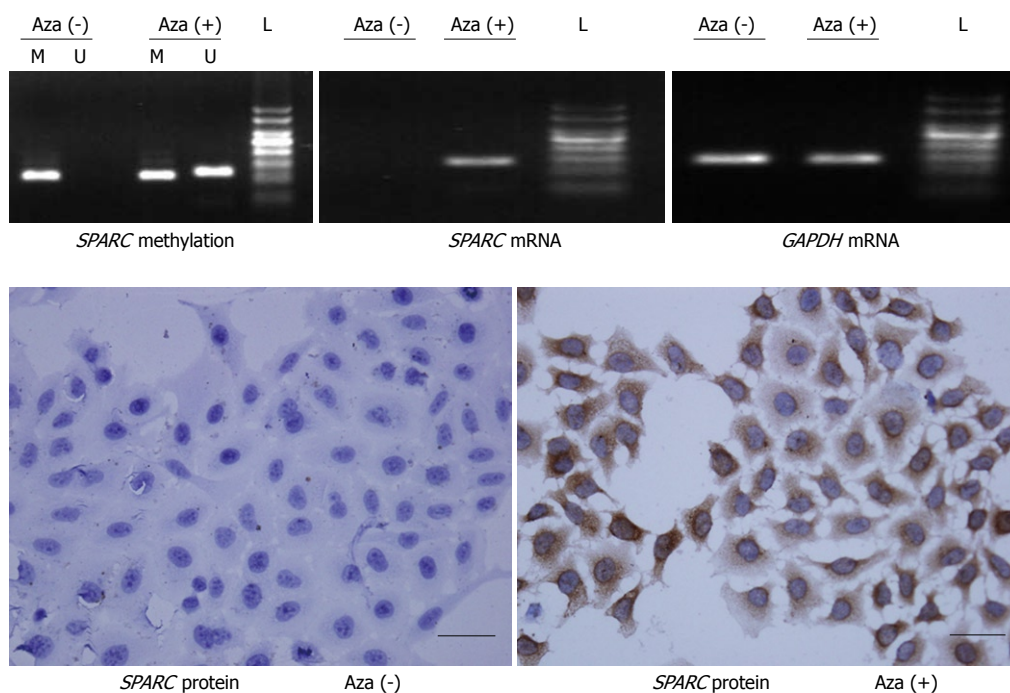
Figure 1 Secreted protein acidic and rich in cysteine methylation and expression in SMMC-7721 cell line. *SPARC*: Secreted protein acidic and rich in cysteine; Aza: 5-aza-2'-deoxycytidine; *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase; M: Methylation; U: Unmethylation; L: 50 bp ladder; Scale bar: 50 µm.

Table 2 Methylation frequencies of secreted protein acidic and rich in cysteine in 60 cases

Tissue	*SPARC* methylation status		*P* value
	Methylated (%)	Unmethylated (%)	
Tumorous	45 (75.00)	15 (25.00)	< 0.001
Nontumorous	7 (11.67)	53 (88.33)	

SPARC: Secreted protein acidic and rich in cysteine.

than 0.05 was defined as being statistically significant. Analyses were performed with SPSS V 13.0 software for Windows (SPSS, Chicago, United States).

RESULTS

Methylation status and expression of SPARC in SMMC-7721 cells

We used MSP to measure both methylated and unmethylated segments in the *SPARC* promoter region. The results demonstrated that only the methylated segment was detected in SMMC-7721 cells of the control group. However, both methylated and unmethylated segments were found in the cells after treated with 5-Aza-CdR. These results indicated that *SPARC* was homologously methylated in SMMC-7721 cells and 5-Aza-CdR could convert the methylation status of *SPARC*. RT-PCR revealed that the *SPARC* mRNA expression was absent in the cells without the 5-Aza-CdR treatment, however, the cells treated with the 5-Aza-CdR restored the *SPARC* mRNA expression. Consistently, the immunocytochemical analysis of the cultured cells displayed that the *SPARC* protein expression was restored in the cells previously lacking

of the *SPARC* expression. The concordance between the loss of gene expression and the aberrant methylation suggested that the DNA methylation played a causal role in the loss of the *SPARC* expression in SMMC-7721 cells. The representative results are shown in Figure 1.

The bisulfite sequencing displayed that the control cells were methylated at almost all the 18 CpG sites in the 8 clones. On the contrary, most of CpG sites were unmethylated in the cells treated with 5-Aza-CdR. Figure 2 shows the methylation pattern of the *SPARC* promoter in SMMC-7721 cells.

Frequent SPARC hypermethylation in human HCC

We used MSP to evaluate the *SPARC* methylation status of the CpG island in 60 pairs of tissues. Methylation alleles in 75.00% (45/60) of HCC samples were detected, however, only 11.67% (7/60) methylated alleles could be found in the corresponding nontumorous tissues. The methylation frequence of *SPARC* in HCC was significantly higher than that in noncancerous liver tissues (Table 2). If methylation was used as an indicator for distincting HCC from nontumorous tissues, the sensitivity, specificity and accuracy were 86.54%, 77.94% and 81.67%, respectively. To validate the accuracy of MSP, we randomly chose the PCR products of methylation or unmethylation for sequencing. The results were according to the PCR aim segments. The representative results of PCR and sequencing are demonstrated in Figure 3.

Correlation between SPARC methylation and mRNA expression

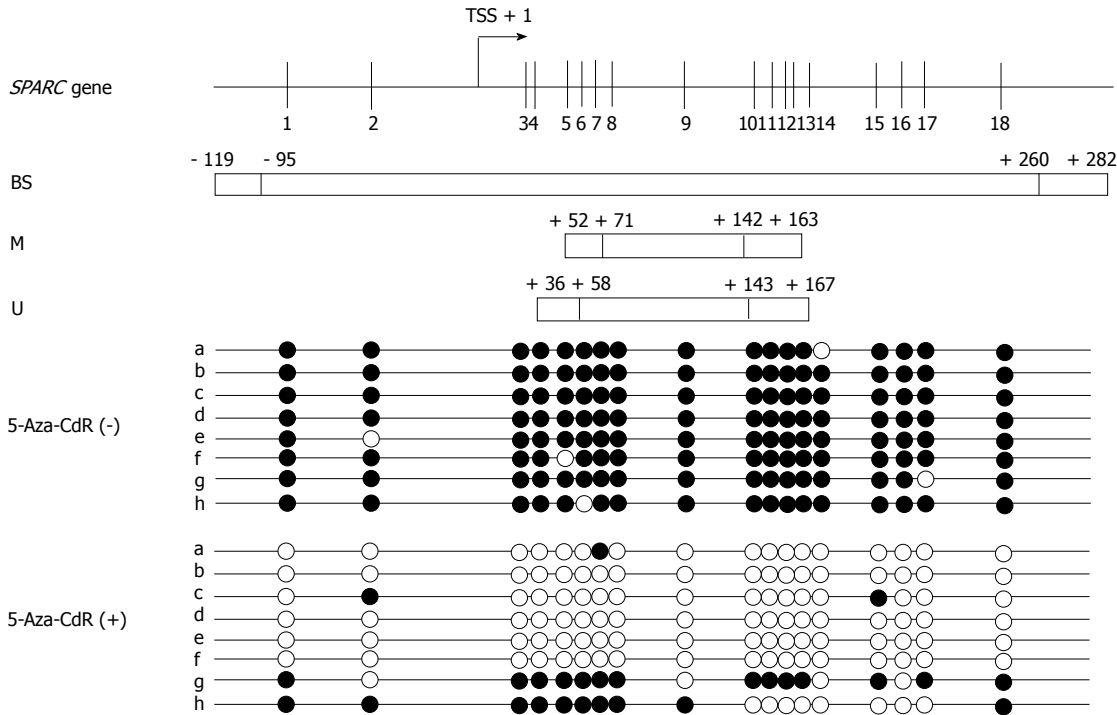The expression of *SPARC* mRNA was examined in 60 pairs of HCC and nontumorous tissues by quantitative

Figure 2 Bisulfite sequencing of secreted protein acidic and rich in cysteine in SMMC-7721 cell line. *SPARC*: Secreted protein acidic and rich in cysteine; TSS: Transcription start site; BS: Bisulfite sequencing; M: Methylation; U: Unmethylation; 5-Aza-CdR: 5-aza-2'-deoxycytidine; 1-18: CpG sites; - 119 to - 95, + 260 to + 282, + 52 to + 71, + 142 to + 163, + 36 to + 58, + 143 to + 167: Polymerase chain reaction primers position; Black dots: Methylation; Blank rings: Unmethylation.
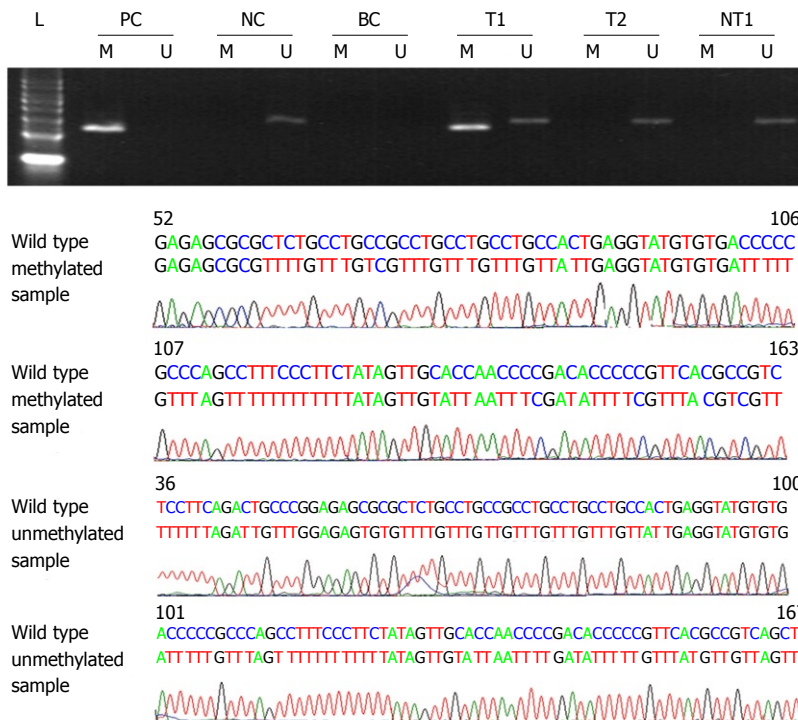

Figure 3 Representative results of methylation specific polymerase chain reaction analysis and sequencing in tissues. L: 50 bp ladder; PC: Positive control; NC: Negative control; BC: Blank control; M: Methylation; U: Unmethylation; T: Hepatocellular carcinoma tissue; NT: Nontumorous tissue.

RT-PCR. Most of primary HCC tissues (65.00%, 39/60) showed a lower expression level when compared with their corresponding nontumorous livers (Figure 4A). Moreover, the median of relative expression was statistically different between the methylated and unmethylated *SPARC* samples of HCC ($P = 0.040$) (Figure 4B). The methylated samples had a lower median of expression.

Methylation and protein expression

The protein expression of *SPARC* was examined in 23 pairs of HCC and nontumorous tissues by immunostaining. The positive frequency of tumor cells in HCC was relatively lower than that of liver cells in nontumorous tissues, but there was no statistical significance between two groups (Table 3). We divided all 46 samples into
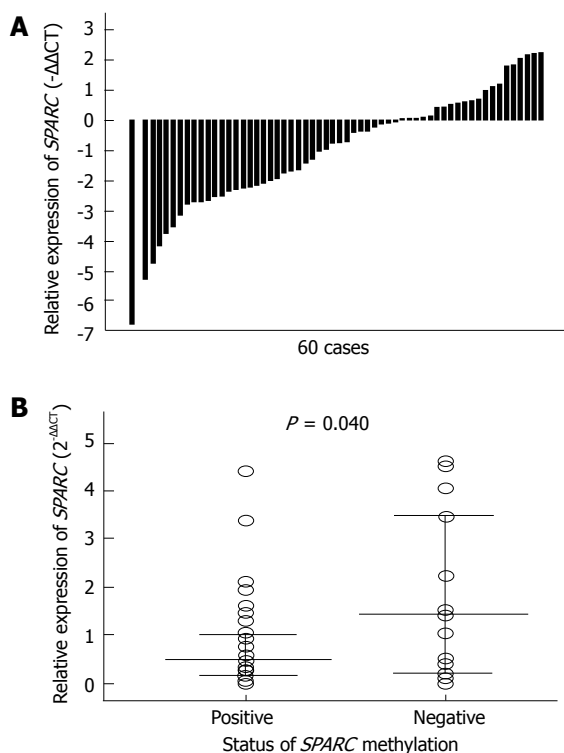
Figure 4 Expression of secreted protein acidic and rich in cysteine mRNA in hepatocellular carcinoma. Horizontal lines represent the median, and range indicates a 25%-75% quartile. *SPARC*: Secreted protein acidic and rich in cysteine.

Table 3 Protein expression frequencies in 23 pairs of samples

| Tissue | n | Protein expression | | P value |
		Positive (%)	Negative (%)	
Tumorous	23	12 (52.2)	11 (47.8)	0.552
Nontumorous	23	14 (60.9)	9 (39.1)	

Table 4 Association of secreted protein acidic and rich in cysteine methylation with protein expression

| *SPARC* | n | Protein expression | | P value |
		Positive (%)	Negative (%)	
Methylated	14	6 (42.9)	8 (57.1)	0.216
Unmethylated	32	20 (62.5)	12 (37.5)	

SPARC: Secreted protein acidic and rich in cysteine.

methylation and unmethylation groups (14 *vs* 32). There was no statistical correlation between the methylation and the protein expression (Table 4). In some HCC samples, stromal cells around tumor cells showed a positive signal even if the parenchyma cells had no expression of *SPARC*. The representative immunohistochemical staining is shown in Figure 5.

Relationship between methylation and clinical data

We analyzed the association of *SPARC* methylation with clinicopathological parameters in patients with HCC.

Table 5 Correlation between methylation status and clinico-pathological data

Parameters	n	Methylated	Unmethylated	P value
Age (yr)				0.766
> 53	30	23	7	
≤ 53	30	22	8	
Gender				0.835
Male	51	38	13	
Female	9	7	2	
Tumor size (cm)				1.000
≤ 5	20	15	5	
> 5	40	30	10	
Virus infection				0.661
HBV or HCV	52	40	12	
Negative	8	5	3	
Liver function				1.000
Child-Pugh A	46	35	11	
Child-Pugh B	14	10	4	
AFP (μg/L)				0.125
≤ 400	35	29	6	
> 400	23	15	8	
Tumor number				0.174
Single	35	24	11	
Multiple	25	21	4	
Vascular invasion				0.122
Positive	22	19	3	
Negative	38	26	12	
Edmondson classification				0.019
I / II	21	12	9	
III /IV	39	33	6	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-fetal protein.

There was significant association between the methylation status and the pathological class. The *SPARC* methylation was more frequently observed in cases with a high pathologic grade (33 of 39, 84.6%) than in those with a low grade (12 of 21, 57.1%) (Table 5). However, there was no statistically significant correlation between the methylation status and other clinicopathologic factors.

Prognostic value of SPARC methylation in HCC

We also divided all cases into two groups according to the methylation status of *SPARC* to determine whether this factor had prognostic value. The disease free survival between the two groups had no statistical difference. Patients whose primary tumors exhibited *SPARC* methylation had a lower overall survival rate after resection (28.0 mo *vs* 41.0 mo, *P* = 0.043, Table 6 and Figure 6). Five clinicopathological factors and methylation status of *SPARC* found to be prognostic on the univariate analysis were entered into a multivariate model to identify independent predictors of overall survival. The Cox's multivariate proportional-hazard model indicated that the factors significantly affecting overall survival were tumor size, AFP level and *SPARC* methylation (Table 7).

DISCUSSION

In this current study, we determined the methylation status of *SPARC* gene promoter in SMMC-7721 cell line
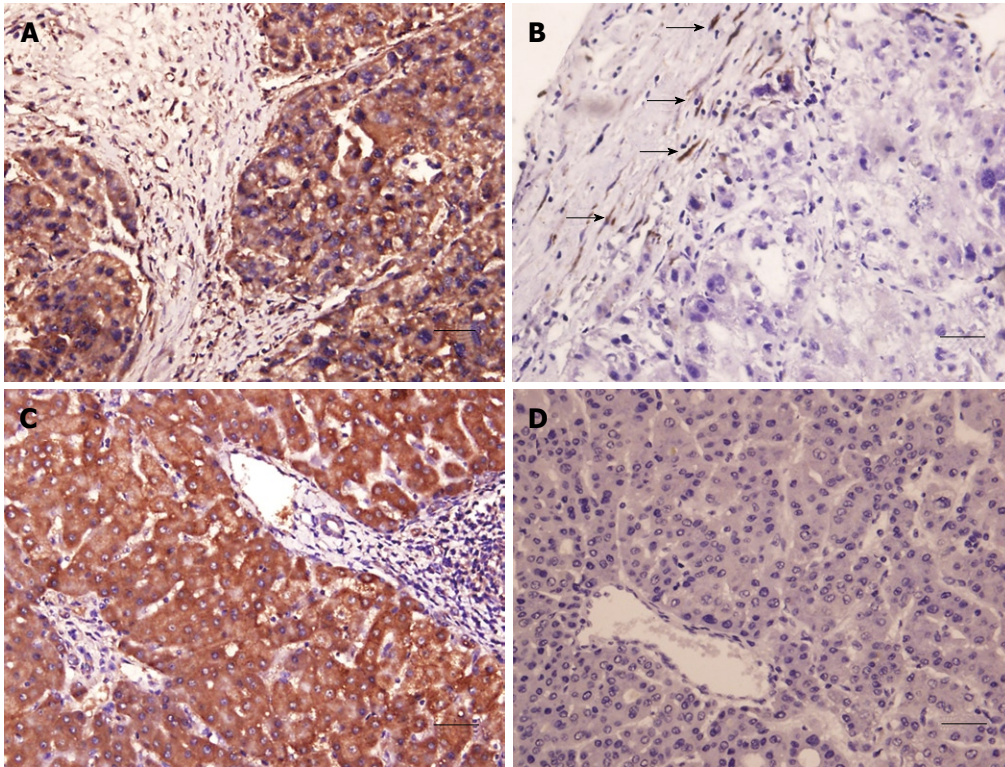
Figure 5 Immunohistochemical analysis of secreted protein acidic and rich in cysteine expression. A: A tumor with positive staining; B: A tumor with negative result, but stromal tissues with positive signal (arrows); C: Nontumorous tissues with positive staining; D: Nontumorous tissues with negative staining; Scale bar: 50 μm.
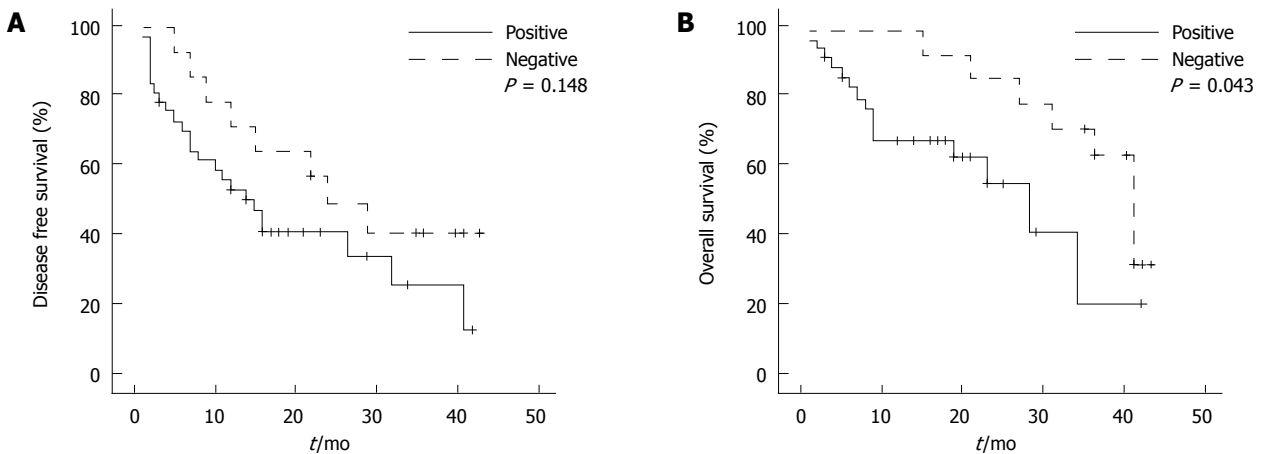

Figure 6 Disease free (A) and overall (B) survival analysis of patients with different secreted protein acidic and rich in cysteine methylation status.

Table 6 Survival analysis of patients with different methylation status

Gene	M/U	*n*	Disease free survival				Overall survival			
			Estimate (mo)	Scope (mo)	Log-Rank	*P* value	Estimate (mo)	Scope (mo)	Log-Rank	*P* value
SPARC	M	37	15.0	9.6-20.4	2.094	0.148	28.0	17.8-38.2	4.096	0.043
	U	14	24.0	12.6-35.5			41.0	36.5-45.5		

SPARC: Secreted protein acidic and rich in cysteine; M: Methylation; U: Unmethylation.

and HCC tissues. The data suggested that in SMMC-7721 cell line, hypermethylation of the promoter was an important mechanism for *SPARC* downregulation, which was most likely involved in the development and progression of HCC. Moreover, the methylation frequency of *SPARC* was significantly higher in the HCC tissues than in the corresponding nontumorous tissues. The hypermethylation of *SPARC* was associated with pathological class and patients without *SPARC* methylation had higher rates of overall survival after resection. Our results showed that methylation of *SPARC* could be further evaluated as a tumor marker for the diagnosis and

Table 7 Cox regression model of overall survival

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P* value	RR	95% CI	*P* value
Methylation						
Positive	2.672	0.999-7.147	0.044	3.207	1.290-7.975	0.012
Negative	1			1		
Tumor size (cm)						
> 5	5.293	1.560-17.959	0.008	8.045	2.125-30.456	0.002
≤ 5	1			1		
AFP (μg/L)						
> 400	3.306	1.421-7.694	0.006	7.105	1.798-28.080	0.005
≤ 400	1			1		
Age (yr)						
> 53	0.663	0.279-1.576	0.353			
≤ 53	1					
Gender						
Male	1.104	0.373-3.266	0.859			
Female	1					
Tumor number						
Multiple	3.330	1.440-7.704	0.005			
Single	1					
Vascular invasion						
Positive	2.776	1.186-6.502	0.019			
Negative	1					
Edmondson classification						
I / II	0.379	0.147-0.982	0.046			
III / IV	1					

AFP: Alpha-fetal protein; RR: Relative risk.

prognosis of HCC.

In some tumor cell lines, aberrant methylation of *SPARC* has been tested. Functional studies have shown that methylation of *SPARC* could induce gene silence and possess tumor suppressing effects[24-26]. Transcription factors were incapable of binding to the methylated DNA of their recognition sequences, therefore, the gene transcription was blocked[24,25]. However, the demethylating agent could convert the methylation status and restore the gene expression. *SPARC* involved in the occurrence and development of certain cancers[27-31]. In concordance with these studies, we observed that the loss of *SPARC* expression correlated with the aberrant methylation and this loss of expression could be rescued by the demethylating agent 5-Aza-CdR. These data suggested that hypermethylation of the promoter is also an important mechanism for *SPARC* inactivation in SMMC-7721 cell line. The results of our DNA bisulfite sequencing of the *SPARC* promoter also displayed that 5-Aza-CdR could convert the methylation status and affect the expression of *SPARC*.

We observed that *SPARC* methylation occurred more frequently in HCC tissues than in nontumorous tissues. We tested the same segments of putative CpG island near the transcription start site in HCC samples, and compared with the previous groups[15,32]. The results showed that *SPARC* methylation was also a relatively higher frequent incident in HCC and the sequencing results validated that there were high-density methylated CpG sites in the amplified region. The distinct methylation status of *SPARC* gene in the benign and malignant tissues was the prereq-

uisite to determine it as an effective molecular biomarker. *SPARC* could discriminate HCC from the nontumorous tissues with a high sensitivity and a specificity, suggesting that *SPARC* methylation may be a promising epigenetic biomarker for the assistant diagnosis of HCC.

In this study, we observed that 65.0% of the HCC samples showed a relatively lower expression level of *SPARC* mRNA compared with the nontumorous tissues. On the contrary, previous groups have reported that *SPARC* was overexpressed in HCC tissues as compared with the nontumorous tissues, nevertheless, *SPARC* mRNA and protein were mainly detected in the tumor capsule, and fibrous bands within HCC[26,33]. *SPARC* was strongly expressed by the stromal myofibroblasts of HCC[26]. In our study, except for different patient population, we used exclusively tumors with more than 80% of epithelial tumor cells to test the *SPARC* mRNA expression, which could minimise the potential contamination of stromal cells in HCC. Some studies in other cancers have revealed aberrant hypermethylation of the *SPARC* promoter to be responsible for low levels of *SPARC* expression[15,16]. In concordance with these studies, we found that the *SPARC* expression of samples with methylation was significantly lower than that without methylation. Although there were other possible mechanisms for the downregulation of the *SPARC* expression, the concordance between the mRNA expression and the DNA methylation indicated that the gene was downregulated, at least partially, through the DNA methylation in HCC. We found no significant correlation between the *SPARC* protein expression and the DNA methylation. The regulation of the translation process or the degradation of protein might also influence the *SPARC* protein abundance in HCC tissues. On the other hand, the *SPARC* protein might be variably expressed by the heterogeneous hypermethylation in one allele of tumor cells. But, interestingly, we also found the *SPARC* expression in the stromal cells in HCC even though the tumor cells had a negative signal, which was accordant with the report[33].

We demonstrated that the pathological class was the only clinicopathological variable associated with the *SPARC* methylation and patients with the *SPARC* methylation tended to have a poorer overall survival after resection in this study. It may be explained by the function of this gene, which was involved in the tumor progression. *SPARC* could inhibit the progress of tumor by restraining the angiogenesis and affecting the extracellular matrix production[34-36]. Our results suggested a potential clinical use of *SPARC* methylation as a prognostic marker in patients with HCC. Because *SPARC* methylation was a kind of DNA marker, it will be possible to detect the status of *SPARC* methylation in peripheral blood in the future, which might be more convenient and less traumatic than using the pathological tissues. However, since the number of patients in this study is relatively small, these findings need to be verified in a study with more patients and a longer follow-up period.

In conclusion, the results in this study indicated that

SPARC promoter hypermethylation in HCC was most likely related to a disease state, which may provide potential diagnostic or predictive markers of this disease.

COMMENTS

Background
Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. The development of biomarkers for early diagnosis and accurate prognosis of HCC is important for improving patients' survival. Aberrant DNA methylation of tumor suppressor genes could be used as a new marker for HCC in the future.

Research frontiers
It has been reported that secreted protein acidic and rich in cysteine (*SPARC*) has tumor suppressing properties to some cancers. Moreover, the *SPARC* promoter methylation is an important factor in the carcinogenesis of these cancers and may be a promising epigenetic marker for them. However, up to date, there have been few reports about the methylation status in HCC. In this study, the authors detected the status of *SPARC* methylation in HCC and estimated its clinical implication.

Innovations and breakthroughs
This is the first study to report that *SPARC* hypermethylation is a high frequent event in HCC. The downregulation of the *SPARC* mRNA expression in HCC is correlated with the *SPARC* methylation. The patients with methylated *SPARC* had a poorer overall survival than those without methylated *SPARC*.

Applications
The results in this study indicated that *SPARC* hypermethylation in HCC is most likely related to a disease state, which might be helpful for finding potential diagnostic or predictive markers of this disease.

Peer review
This is a good descriptive study in which authors investigate the methylation status of *SPARC* in HCC and evaluate its clinical implication.The results are interesting and suggest aberrant methylation is an important mechanism for *SPARC* inactivation in HCC and *SPARC* methylation may be a promising biomarker for the diagnosis and prognosis of HCC.

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