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Original Article

Promoter hypermethylation mediates the down-regulated expression of ADAMTS9 and is associated with the prognosis in hepatocellular carcinoma

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Abstract: This study aims to evaluate the methylation of a disintegrin and metallo-proteinase with thrombospondin type 1 motif, 9 (ADAMTS9) promoter and its effect on the protein expression in hepatocellular carcinoma (HCC). The methylation of the ADAMTS9 promoter in HCC cell lines Hep-G2 and SMMC-7721 and in tissues from 132 cases of HCC, the corresponding adjacent tissues, and 15 normal hepatic tissues was detected by methylation specific polymerase chain reaction (MSP). Expression of ADAMTS9 in the tissues from 78 cases of HCC and the corresponding pericarcinous tissues was tested by immunohistochemistry. Methylation or semi-methylation of the ADAMTS9 promoter was found in the Hep-G2 and SMMC-7721 cell lines. The positive rate of methylation in HCC tissues (66.67%) was significantly higher than in the adjacent tissues (29.55%) and normal tissues (0%, *P*=0.000). The positive rate of ADAMTS9 methylation was associated with partial tumor thrombus (PTT) (*P*=0.029) and the clinical stage of disease (*P*=0.008). However, the positive rate of ADATMS9 expression in cancer tissues (32.89%) was significantly lower than that in adjacent liver cirrhotic tissues (71.05%) and normal liver tissues (90%, *P*=0.000). The rate of ADAMTS9 methylation was inversely related to that of its gene expression. Patients with positive ADAMTS9 methylation had worse survival outcomes than the negative group (*P*=0.002). COX multivariate analysis indicated that ADAMTS9 methylation was an independent prognostic factor in predicting postoperative survival of HCC patients (*P*=0.013). Aberrant methylation is an important mechanism for ADAMTS9 inactivation in HCC. ADAMTS9 methylation may be a promising biomarker for HCC prognosis.

Keywords: HCC, ADAMTS9, methylation, MSP, prognosis

Introduction

Hepatocellular carcinoma (HCC) is a common malignancy that seriously endangers human health and is the second leading cause of cancer death in China [1, 2]. Although the main cause of HCC has been identified, the molecular mechanism of the pathogenesis of HCC has not been elucidated. It is generally believed that a number of genetic changes are involved in the occurrence and development of HCC, such as chromosomal abnormalities, gene amplification and mutation, and epigenetic changes [3]. Previous studies showed that the recurrence of epigenetic changes in HCC, for example, the hypermethylation of CpG, was also an important mechanism of anti-oncogene inactivation [4]. Methylation of CpG in the anti-oncogene promoter is an early event in the occurrence of HCC, and it is associated with the prognosis [5].

A disintegrin and metallo-proteinase with thrombospondin motifs (ADAMTS) family comprise new types of metalloproteinase that are dependent on Zn^{2+} and widely exist in mammals. Nineteen members of the ADAMTS family were found in humans. The known functions of ADAMTS family include dissolution of the extracellular matrix, regulating hemostasis and adjustment of angiogenesis [6], and it also plays an important role in the occurrence and development of tumors [7]. A disintegrin and metallo-proteinase with thrombospondin type 1 motif, 9 (ADAMTS9) is a new member of the ADAMTS family, and has been shown to be a

new tumor-inhibiting factor in the growth of esophageal squamous cell carcinoma [8, 9]. Hypermethylation of the ADAMTS9 gene promoter region occurs in the tissues of nasopharyngeal carcinoma, gastric cancer, colon cancer, esophageal squamous cell carcinoma, and pancreatic cancer, and it is related to the occurrence and development of these cancers [10, 11]. However, the effect of methylation of ADAMTS9 on HCC has not yet been defined.

In this study, the methylation status of the DNA promoter region of the ADAMTS9 gene and its role in the expression of ADAMTS9 protein in HCC cell lines and fresh liver tissues were evaluated. In addition, an analysis was performed to examine the influence of the ADAMTS9 gene on the occurrence and development of HCC, and to explore the importance of ADAMTS9 gene methylation in predicting the progression and prognosis of HCC.

Materials and methods

Cell culture and 5-Aza-2'-deoxycytidine treatment

SMMC-7721 and Hep-G2 cell lines were obtained from the Cancer Research Institute of Beijing (Beijing, China). The Hep-G2 cell line was cultured in RPMI-1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum and 100 U/mL each of penicillin and streptomycin. The SMMC-7721 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum and 100 U/mL each of penicillin and streptomycin. The two cell lines were incubated in 5% CO_2 at 37°C. When the cell number reached 10^6 cells/ml, the cells were digested with 0.25% trypsin-EDTA. Cells were cultured for 24 h after digestion and then the medium was replaced by medium containing 10 μM/mL 5-Aza-2'-deoxycytidine (5-Aza-CdR) (Aza; Sigma-Aldrich, St. Louis, MO, USA). Twenty-four hours later, medium with serum and without 5-Aza-CdR was used to culture the cells for another 72 h. Cells were collected after reaching the logarithmic growth phase.

Patients and clinical specimens

One hundred thirty two patients who underwent surgical resection for HCC in the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between April 2009 and January 2011 were enrolled in this study. There were 113 males and 19 females, ages 23-78 years old (54.29±9.78). The range of the diameter of tumors studied was 0.5~25 cm, with 123 tumors of grade A, and 9 of grade B and C. According to American Joint Committee on Cancer (AJCC) standard [12], among the 132 patients, there were 40 cases of stage I, 76 cases of stage II, 15 cases of stage III, and 1 case of stage IV disease. Patients in the study were not treated with other adjuvant therapies. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Fourth Hospital of Hebei Medical University. Written informed consent was obtained from all participants.

Medical history, laboratory findings, and pathological data of patients were collected, and then the patient status was confirmed in a follow-up telephone call or letter during the follow-up period. Survival time was calculated from the first day after operation to death or the end of follow-up period, setting months as the unit of measure. The deadline of the follow-up period was February 2013.

A total of 279 specimens including 132 HCC tissues with corresponding adjacent cirrhotic tissues, and 15 normal liver tissues were obtained. Adjacent liver cirrhotic tissue was taken 2 cm from cancerous tissue, immediately frozen in liquid nitrogen and then transferred to a -80°C freezer for storage. Specimens were embedded with paraffin and stained with hematoxylin-eosin (HE). Two doctors of pathology who provided a histodiagnosis examined the stained tissues. Among the tissues examined, there were 25 tissues that exhibited high differentiation, 21 that exhibited mild differentiation, and 86 cases that exhibited poor differentiation or were undifferentiated.

DNA extraction and sulfite treatment

The genomic DNA was extracted from the cell lines or tissue samples using a DNA extraction kit (Promega, Madison, USA). Pre-cooling nuclear lysate (600 μl) was added to 10-20 mg tissue samples, the samples were homogenated for 10 s with the grinder, lysates was transferred into a 1.5 ml Eppendorf (EP) tube and then incubated for 15-30 min at 65°C. Protein

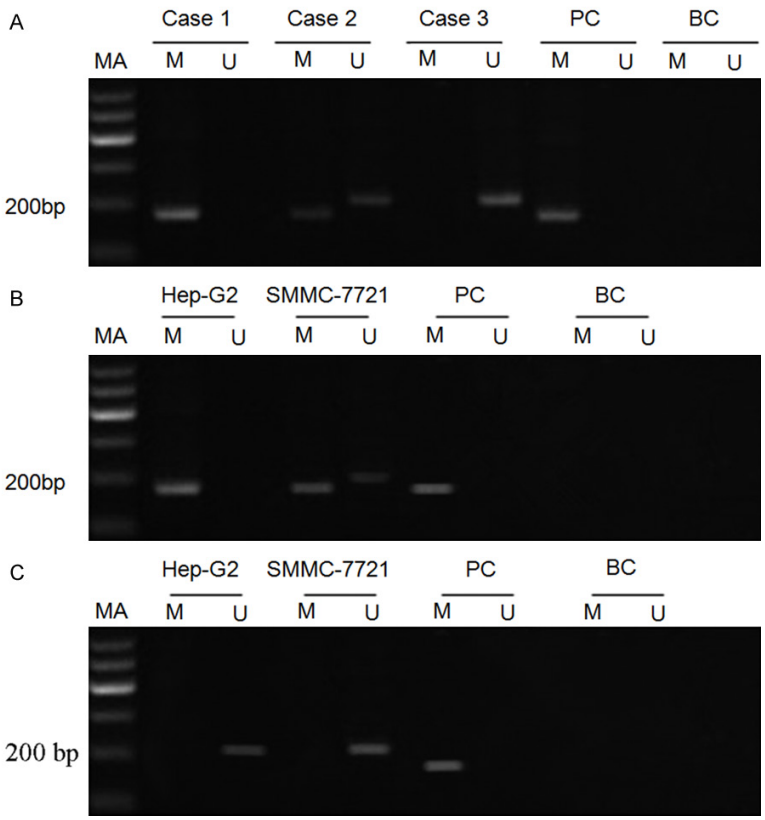

Figure 1. ADAMTS9 CpG island methylation analysis in hepatocarcinomar cell line. A: The methylation status of tumor suppressor genes ADAMTS9. Case 1: Methylated; Case 2: Hemi-Methylated; Case 3: Unmethylated. MA: Marker; PC: Positive control; BC: Blank control; M: Methylated; U: Unmethylated. B: The methylation status of tumor suppressor genes ADAMTS9 of Hep-G2 and SMMC-7721 lines before treated with 5-Aza-cdR. PC: Positive control; BC: Blank control; MA: Marker; M: Methylated; U: Unmethylated. C: The methylation status of tumor suppressor genes ADAMTS9 of Hep-G2 and SMMC-7721 lines after treated with 5-Aza-cdR. PC: Positive control; BC: Blank control; MA: Marker; M: Methylated; U: Unmethylated.

precipitation liquid (200 μl) was added, and the tube was put it on ice for 5 min after mixing on a vortex mixer. The samples were centrifuged for 4 min at 13000-16000×g, the supernatants were transferred into microfuge tubes containing 600 μl isopropanol, and the samples were mixed gently. The samples were centrifuged at room temperature for 1 min until white precipitates of DNA were visible, and then the supernatants were carefully discarded. Seventy percent ethanol (600 μl) was added to each sample and samples were centrifuged (13000-16000×g) at room temperature for 1 min. The supernatant liquid was discarded, samples were dried on the filter paper, 100 μl DNA rehydration solution was added, and DNA samples were incubated at 4°C overnight. DNA samples were stored at -2°C.

DNA solution (7 μg) and 11 μl distilled water were added to a 1.5 ml EP tube and placed in a water bath at 120°C for 10 min. Samples were then put immediately on ice for 5 min. The samples were denatured by the addition of 2 μl 3 M NaOH, and incubated in a 42°C water bath for 20 min. Three hundred eighty microliters of DNA processing liquid containing bisulfite and hydroquinone was added into the EP tube, 200 μl of mineral oil was added to the top, and the samples were placed in a waterbath at 50°C in the dark for 16 h. The liquid under mineral oil was removed, and 1 ml of the DNA purified liquid was processed using a DNA Purification Kit (Promega, Madison, step USA) as previous described [13]. Eleven microliters of 3 M NaOH was added into the tube, and the tube was incubated in a water bath at 37°C for 15 min. Ammonium acetate (83 μl) and 485 μl ethanol was added to the EP tube, and the tube was frozen at -80°C for 30 min. The DNA pellet was obtained by centrifugation at 12,000×g for 20 min, and the supernatant was discarded. The pellet was washed with 200 μl 70% ethanol, centrifuged at 14000×g for 20 min and the supernatant was discarded. After drying on filter paper, the DNA precipitation was dissolved by adding 30 μl TE (1 M Tris-HCl Buffer and 0.5 M EDTA) buffer and placed at 4°C overnight. The genomic DNA was stored at -20°C for further experimentation within seven days or at -80°C for use within one year.

Detection of ADAMTS9 methylation

Methylation-specific PCR (MSP) was carried out for 38 cycles in a thermocycler (Mastercycler5333) with annealing temperatures of 59.2°C for the methylated reaction and 57.1°C for the unmethylated reaction. Methylation-spe-

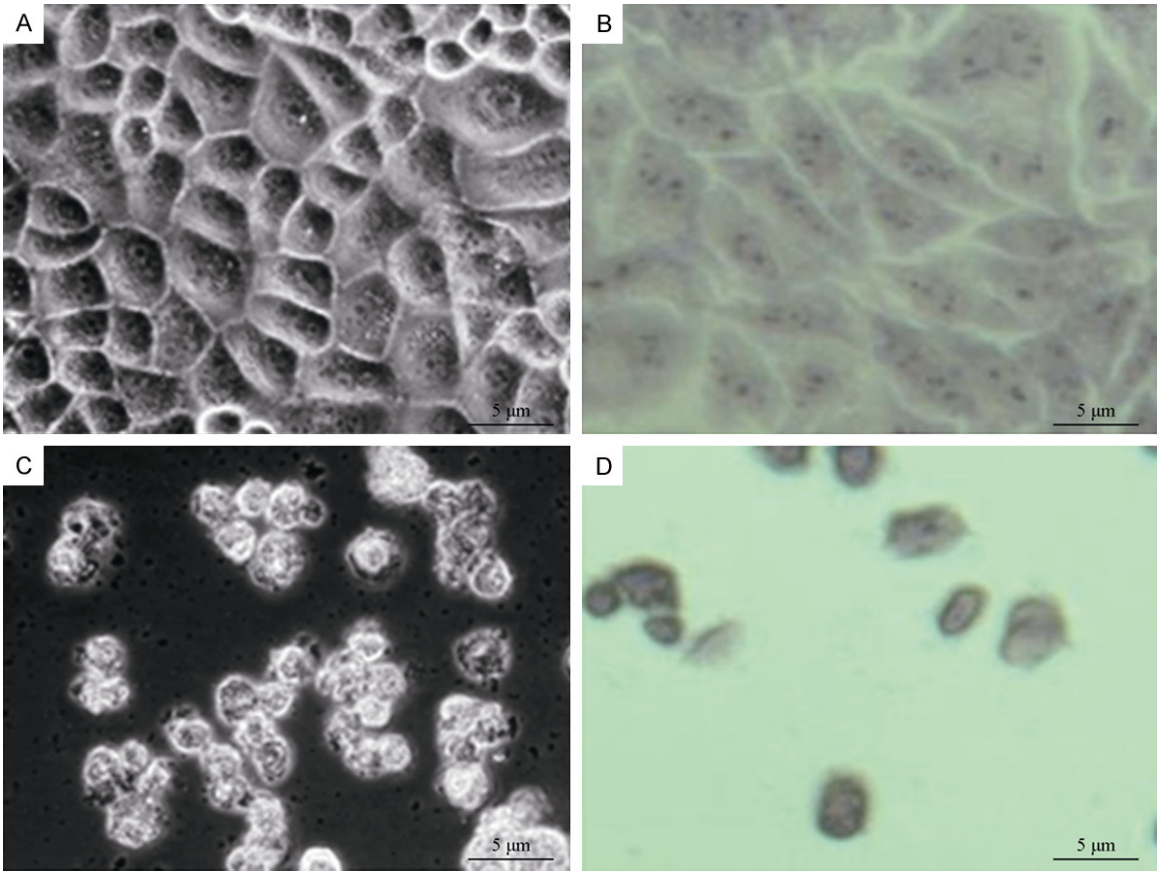

Figure 2. Morphological changes of Hep-G2 and SMMC-7721 cells before and after treated with 5-Aza-cdR. Before treated with 5-Aza-CdR, Hep-G2 (A) and SMMC-7721 (B) cells were round or spindle type and the nuclei were round or oval, the nucleolus was clear and patina was rich. It was easy to see the mitotic cells. After treated with 5-Aza-CdR, the number of Hep-G2 (C) and SMMC-7721 (D) cells was less than that of the control, shrinkage was visible and the lucency decreased. Nucleolus was not clear, and cell lysis fragments can be seen in the medium.

cific primers were: FBP1-MF-5'-TTCGGTTAGTATAGGGCGTTTGGCG-3' and FBP1-MRR-5'-AAAATCTCGCTACGAAAATCCCGTC-3'. Unmethylation-specific primers were: FBP1-UF-5'-TGTTTGTTTGTTGGGTATTATGTGG-3' and FBP1-UR-5'-CCAACTTTTAACTTTAAAAATCACT-3'. Human methylated and unmethylated DNAs were used as controls to verify the specificity of the primers.

Immunohistochemistry

The specimens were fixed in 10% formalin and embedded in paraffin. Five serial 4 μm sections were cut from the tissue blocks. The deparaffinized sections were stained with the anti-ADAMTS9 antibody (Sigma-Aldrich, St Louis, MO, USA) at a dilution of 1:250 overnight at 4°C followed by incubation with a biotinylated secondary anti-mouse IgG antibody for 1 h at room temperature. The sections were subsequently incubated with horseradish peroxidase (HRP)-conjugated streptavidin and were developed using 3,3'-diaminobenzidine (DAB). The evaluation of ADAMTS9 staining was performed by two pathologists with no background on the clinical data of patients as previously described [14]. The percentage of positive tumor cells was graded as follows: 0=0%, 1=1-25%, 2=26-50%, 3=more than 50%. Immunostaining intensity was rated as follows: 0=none, 1=weak, 2=moderate, 3=intense. The specimen was considered immunopositive if the score when multiplying the percentage of positive cells with the intensity score is equal to or greater than 1.

Statistical analysis

Using SPSS13.0 statistical software package (SPSS Inc., IL, USA, Chicago) the following analyses were performed: measurement data using t test or non-parametric test, count data using χ^2 test or Fisher exact probability method, sur-

Table 1. Relationship between the ADAMTS9 DNA promoter methylation and the clinical characteristics in patients with hepatocellular carcinoma

Clinical information	ADMATS9 methylation status		Positive rate (%)	X^2	*P*
	+	-			
Gender				1.506	0.220
Male	73	40	64.60		
Female	15	4	78.95		
Age (year)				1.840	0.175
≤55	45	17	72.58		
>55	43	27	61.43		
Child classification					0.717
A	81	42	65.85		
B+C	7	2	77.78		
AFP (ug/L)				0.627	0.428
≤200	62	34	64.58		
>200	26	10	72.22		
Tumor size (cm)				0.567	0.452
<5	17	11	60.71		
≥5	71	33	68.27		
Portal vein tumor embolus				4.774	0.029
Yes	37	10	78.72		
No	51	34	60.00		
Tumor number				0.027	0.869
Single	73	37	66.36		
Multiple	15	7	68.18		
HBsAg				0.229	0.632
Positive	71	37	65.74		
Negative	17	7	70.83		
Tumor metastasis				0.462	0.497
Yes	27	11	71.05		
No	61	33	64.89		
Pathology differentiation level				0.025	0.875
Well differentiation	17	8	68.00		
Moderate-poor differentiation	71	36	66.36		
Clinical stage				6.970	0.008
I~II	82	34	70.69		
III~IV	6	10	37.50		

vival analysis using Kaplan-Meier method, single factor analysis using Log-rank test, and multivariate analysis using COX model. $P<0.05$ was considered as statistically significant.

Results

DNA methylation of ADAMTS9

The DNA methylation of ADAMTS9 gene had three forms: fully methylated, semi methylated and completely non-methylated (**Figure 1A**).

The growth status of HCC cells and the methylation status of ADAMTS9

Before treatment with 5-Aza-CdR, Hep-G2 and SMMC-7721 cells were morphologically round or spindle type, the nuclei were round or oval, the nucleolus was clear and patina was rich. It was easy to see the mitotic cells. After treatment with 5-Aza-CdR, the number of cells was less than that of the control, shrinkage was visible and the lucency was decreased. The nucleolus was not clear, and cell lysis fragments could be seen in the medium (**Figure 2**).

Before adding 5-Aza-CdR, the DNA of HCC cell lines Hep-G2 and SMMC-7721 was detected by MSP, which showed the methylation band of ADAMTS9. Hep-G2 cell DNA had a fully methylated band, and SMMC-7721 cell DNA had a semi-methylated band (**Figure 1B**). After adding 5-Aza-CdR, ADAMTS9 DNA methylation was not detected and a non-methylated band could be seen in two cell lines (**Figure 1C**).

Comparison of methylation status of ADAMTS9 promoter region in different liver tissues

Positive rates of ADAMTS9 methylation in 132 HCC tissues and adjacent liver cirrhotic tissue was 66.67% (88/132) and 29.55% (39/132), respectively. The methylation of the DNA promoter region was not found in normal liver tissues. Comparison of positive rates in these three groups was considered as statistically significant ($P=0.000$). The positive rate of

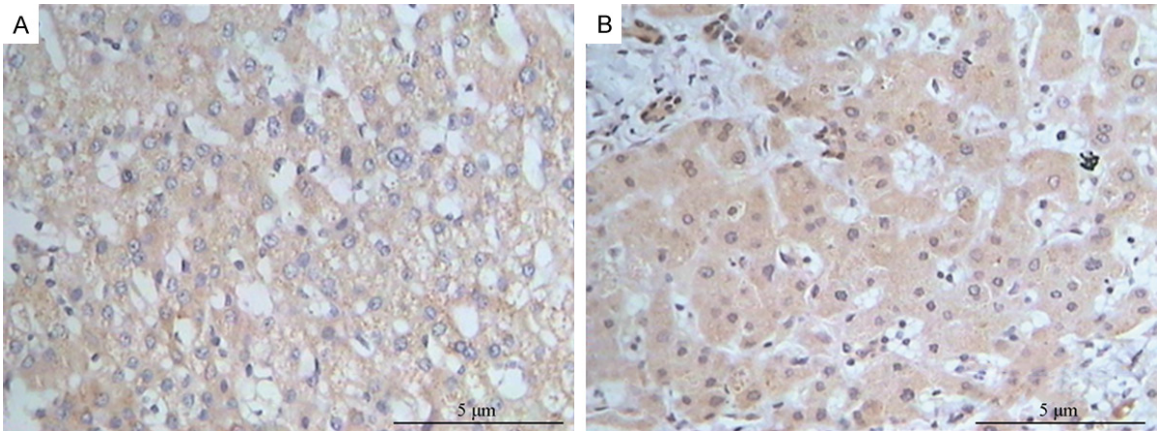

Figure 3. ADAMTS9 expression by immunohistochemical detection in paraffin embedded HCC and adjacent tissues. A: Cytoplasmic expression of ADAMTS9 in more than 50% cells with intense stain in carcinoma (original magnification ×400); B: Cytoplasmic expression of ADAMTS9 in more than 50% cells with moderate stain in adjacent tissue of HCC (original magnification ×400).

ADAMTS9 methylation in HCC was significantly higher than that in adjacent liver cirrhotic tissue (P=0.000).

Comparison of ADAMTS9 gene methylation status and clinical pathological characteristics of patients with HCC

The positive rate of ADAMTS9 methylation in patients with portal vein tumor thrombus was higher than that of patients without portal vein tumor thrombus; the difference between them was statistically significant (78.72% vs 60.00%, P=0.029). The positive rate of DNA methylation in patients with stage I-II was higher than that in patients with stage III-IV (70.69% vs 37.50%, P=0.008).

The positive rates of ADAMTS9 methylation were not statistically significant (P>0.05, **Table 1**) in patients with different groups of clinical and pathological characteristics such as gender, age, Child-Pugh grade, α-fetoprotein (AFP) level, tumor size, tumor number, HBsAg (hepatitis B virus surface antigen), with or without metastasis and pathological differentiation.

Expression of ADAMTS9 protein in HCC tissues

Seventy-six HCC tissues and corresponding adjacent cirrhotic tissues were examined for expression of ADAMTS9 protein by immunohistochemistry. ADAMTS9 gene protein expression was determined by the appearance of a brown yellow flake or granular stain in the cytoplasm (**Figure 3**). In 76 cases of HCC, the positive expression rates of the protein was 32.89%. In the 76 samples of adjacent tissue, 54 tissues showed ADATMS9 gene protein expression, and the expression rate was 71.05%. In normal liver tissues, 9 tissues exhibited ADATMS9 gene protein expression, and the expression rate was 90%. The difference between these three groups was considered as statistically significant (P=0.000), and the positive rate of normal tissues and tumor adjacent tissues were higher compared to cancerous tissues.

Comparison of ADAMTS9 protein expression in different clinical pathological characteristics

The difference of ADAMTS9 protein expression between patients' gender, age, Child-Pugh classification, AFP, tumor size, number of tumors and the presence of HBsAg was not statistically significant (P>0.05). However, the difference between patients with or without metastasis, patients with high differentiation and low differentiation, and patients with stage I~II and stage III~IV were statistically significant (P=0.041, P=0.003, P=0.046, respectively) (**Table 2**).

Relationship between methylation of ADAMTS9 and expression

In tissues with positive methylation of the ADAMTS9 gene promoter region, protein expression in 7 tissues was observed, (17.07%). In tissues with negative methylation of ADAMTS9 gene promoter region, protein expression was positive in 18 tissues (48.64%.) The difference

Table 2. Relationship between the ADAMTS9 protein expression and the clinical characteristics in hepatocellular carcinoma

Clinical information	ADMATS9 protein expression		Positive rate (%)	X^2	*P*
	+	-			
Gender				0.110	0.740
Male	21	44	32.31		
Female	3	8	27.27		
Age (year)				3.498	0.061
≤55	16	21	43.24		
>55	9	30	23.08		
Child classification				0.777	0.378
A	24	46	34.29		
B+C	1	5	16.67		
AFP (ug/L)				1.125	0.289
≤200	10	27	27.03		
>200	15	24	38.46		
Tumor size (cm)				0.497	0.481
<5	5	14	26.32		
≥5	20	37	35.09		
Portal vein tumor embolus				0.356	0.551
Yes	8	13	38.10		
No	17	38	30.90		
Tumor number				1.082	0.298
Single	18	42	30.00		
Multiple	7	9	43.75		
HBsAg				0.328	0.567
Positive	21	40	34.43		
Negative	4	11	26.67		
Tumor metastasis				4.185	0.041
Yes	4	20	16.67		
No	21	31	40.38		
Pathology differentiation level				9.119	0.003
Well differentiation	9	5	64.29		
Miderate-poor differentiation	16	46	25.81		
Clinical stage				3.987	0.046
I~II	16	43	27.12		
III~IV	9	8	52.94		

between the two groups was statistically significant (*P*=0.036). The rate of ADAMTS9 methylation was inversely related to that of gene expression.

Relationship between the methylation status of ADAMTS9 gene promoter region and the prognosis of survival time

The two-year survival rate of patients with positive methylation of the ADAMTS9 gene promoter region was 29.5%, median survival time was 10 months and mean survival time was 12.50±0.93 months. The two-year survival rate of patients with negative methylation was 50.0%, median survival time was 23 months and mean survival time was 19.61±1.19 months. The difference was statistically significant (*P*= 0.002, **Figure 4A**).

The two-year survival rate of patients with tumor metastasis was 23.8% and was 42% in patients without tumor metastasis. The difference between the rates was statistically significant (*P*= 0.036, **Figure 4B**). Gender, age, Child-Pugh grade, AFP value, tumor size, number of tumors, presence of HBsAg and clinical stages were analyzed. The results showed that there was no statistical significance between the two-year survival rates when these parameters were compared.

In order to analyze the independent prognostic factors related to overall survival of patients with HCC, gender, age, Child-Pugh grade, AFP value, tumor size, number of tumors, presence of HBsAg, metastasis, differentiation degree, clinical stage and methylation of ADAMTS9 gene, an analysis using COX was performed. Results showed that the methylation status of tumor suppressor gene ADAMTS9 was an independent factor for predicting the survival time of patients with HCC (*P*=0.013) (**Table 3**).

Discussion

Previous studies have indicated that ADAMTS9 inhibited tumor growth by inhibiting the formation of blood vessels [13, 15], and the methylation of the ADAMTS9 promoter was associated with the occurrence of cancer [10, 11]. However,

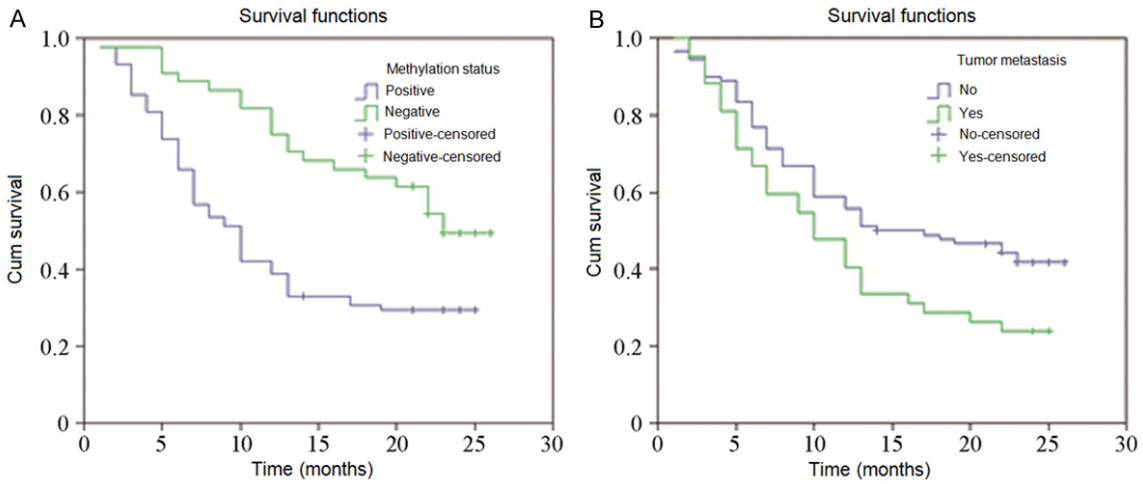

Figure 4. Survival curves for OS of various subgroups HCC patients. A: A significant correlation pattern was detected in patients when categorized into subgroups of positive and negative mathylation status of ADAMTS9 (P=0.002); B: The OS was significantly different in subgroup of patients with metastasis and without metastasis (P=0.036).

Table 3. Multivariate analysis of prognostic factors associated with post-operational survival in HCC patients with the Cox proportional hazard model

Factors	Relative risk	95% CI	*P*-value
Gender	1.210	0.614-2.385	0.583
Age	0.970	0.936-1.005	0.089
Child classification	0.987	0.398-2.445	0.977
AFP	1.334	0.803-2.218	0.266
Tumor size	1.150	0.640-2.066	0.641
Portal vein tumor embolus	1.091	0.665-1.788	0.730
Tumor number	1.077	0.588-1.972	0.809
HBsAg	1.172	0.623-2.204	0.623
Tumor metastasis	1.401	0.855-2.297	0.181
Pathology differentiation level	0.959	0.530-1.733	0.889
Clinical stage	1.431	0.708-2.891	0.319
Methylation	0.497	0.287-0.862	0.013

angiogenesis plays an important role in the occurrence of HCC and is related to the ability of HCC to invade and metastasize [16]. Thus, it is predicted that methylation of ADAMTS9 may have a certain effect on the occurrence, development and prognosis of HCC. The methylation status of HCC cell lines, SMMC-7721, Hep-G2, and fresh HCC tissues with corresponding adjacent tissues was analyzed in this study first. The findings demonstrated that high methylation of ADAMTS9 promoter region might be involved in hepatocarcinogenesis, affect the expression of ADAMTS9 gene, and be related to the prognosis of HCC.

The methylation of ADAMTS9 was detected in 6 gastric cancer cell lines (BGC823, MGC803, AGS, SNU719, YCCEL1 and Kato III) [17] and myeloma cell line KM3 [18]. Being consistent with the above results, our results revealed that the methylation band of ADAMTS9 appeared both in SMMC-7721 and Hep-G2 cell lines, and the methylation bands disappeared after using 5-Aza-CdR which also inhibited the growth of the cells.

Based on the results in the HCC cells, the methylation of ADAMTS9 promoter region in other tissues was further examined. In this study, high methylation rates were observed in 88 cases among 132 cases of HCC (66.67%) while abnormal methylation was found in tissues adjacent to the tumor in only 39 of the 132 cases (29.55%), and the incidence of CpG gene methylation in normal liver tissue was not detected. The incidence of abnormal methylation of ADAMTS9 gene in normal liver tissue, liver cirrhosis, and HCC tissues were gradually increased, suggesting that methylation of ADAMTS9 promoter may play a role in the progression from normal liver tissue to cirrhosis to HCC. Zhang et al. [11] studied tissues and corresponding adjacent tissues from 100 cases of gastric cancer, and 70 cases of pancreatic cancer and found that the positive methylation rate in gastric cancer

was 36% while only 2% in normal gastric tissue and the rate was 57% colon cancer tissues and 1% in normal colonic tissues. Among 70 cases of pancreatic cancer, the positive rate of ADAMTS9 methylation was 47.14%, and 1.43% in the corresponding normal tissues; the positive rate of ADAMTS9 methylation in the three cancer tissues was significantly higher than that in tissues adjacent to the tumor. Du et al. [17] studied the methylation of ADAMTS9 in 72 gastric cancer tissues and 20 normal tissues. The results showed that the positive rate of ADAMTS9 methylation was 29.2%, and methylation of ADAMTS9 did not occur in the normal tissues. Results of this study indicated that the positive rate of ADAMTS9 methylation in HCC was significantly higher than that found in gastric cancer and pancreatic cancer, which demonstrated that the methylation of ADAMTS9 was more specific in HCC. These results suggest that detection of ADAMTS9 methylation can be used as a new indicator of diagnosis and differential diagnosis of HCC.

The relationship between the methylation of the ADAMTS9 gene and expression of protein has not been reported in previous studies. Most researchers were still focused on studying the level of mRNA and studying the relationship between the gene methylation and mRNA expression [19, 20]. Peng et al. [18] found that methylation of ADAMTS9 gene affected the transcription, resulting in the decrease of ADAMTS9 mRNA expression. In the previous studies on other tumor suppressor genes such as APC [21], P16 [22] and RIZ1 [23], it was found that the methylation of the anti-oncogene promoter region could lead to the decrease of gene protein expression. Detection of ADAMTS9 protein expression in liver tissues showed that the positive rate of protein expression was 32.89% in HCC tissues and 71.05% in tissues adjacent to the tumor; the protein expression of ADAMTS9 was down-regulated in HCC tissues. In addition, analysis of the relationship between the methylation and protein expression of ADAMTS9 revealed that in HCC tissues, the positive rate of ADAMTS9 methylation was higher, and the positive rate of protein expression was lower. In tissues adjacent to the tumor, the positive rate of ADAMTS9 methylation was lower, and the positive rate of protein expression was higher. This reverse relationship allows us to speculate that gene silencing caused by the methylation of ADAMTS9 promoter is the reason for the down-regulation of ADAMTS9 expression in HCC tissues. This hypothesis was confirmed in gastric cancer, multiple myeloma, and nasopharyngeal carcinomas. The silencing of ADAMTS9 gene was caused by the methylation of promoter region in cases of gastric cancer, multiple myeloma, and nasopharyngeal carcinomas, which further led to the formation of tumors [10, 17, 18]. In HCC, the epigenetic regulation mechanism of other tumor suppressor genes such as APC [21], P16 [22], RIZ1 [23] were consistent with the results of this study. All these finding showed that the methylation of ADAMTS9 promoter resulted in the occurrence of HCC.

Further analysis of the methylation status of promoter region, ADAMTS9 expression and parameters of clinical pathological parameters showed that the positive rate of ADAMTS9 promoter region methylation were different at different clinical stages and with or without partial tumor thrombus. The positive rate of methylation in patients with partial tumor thrombus was significantly higher than that of patients without partial tumor thrombus; the partial tumor thrombus was one of important factors reflecting the invasion and metastasis of HCC [24], which represented the severity of HCC. The positive rate of methylation of patients at stage I~II was higher than that of patients at stage III~IV, indicating that the methylation of ADAMTS9 promoter region was an early event in the formation of HCC. The difference of ADAMTS9 expression in HCC metastasis, differentiation, and clinical stage was statistically significant and consistent with the results in nasopharyngeal carcinomas where it was found that the expression of ADAMTS9 in the lymph node metastasis group was absent or down-regulated [10]. It was suggested that the methylation of ADAMTS9 promoter region might be involved in the progression of HCC. Our study also revealed that the methylation of ADAMTS9 promoter region was related to the survival rate of HCC patients, which was consistent with the conclusion of gastric cancer studies [17]. COX analysis demonstrated that the methylation of ADAMTS9 promoter region was an independent risk factor of poor prognosis in patients with HCC, suggesting that the methylation of ADAMTS9 could be used as a prognostic indicator in patients with HCC.

In summary, we determined that ADAMTS9 is a new tumor suppressor factor in HCC, and its inactivation was associated with the methylation of promoter region. The methylation of ADAMTS9 gene is related to the biological indicators used in the prognosis of the patients with HCC. The detection of ADAMTS9 gene methylation is expected to be a new target for the diagnosis and prognosis of HCC.

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Disclosure of conflict of interest

None.

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