Downregulation of SM22α protein by hypermethylation of its promoter in colorectal cancer

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Abstract. Silencing of tumor suppressor genes by hypermethylation in gene promoter regions is a crucial mechanism in carcinogenesis. Gene methylation may be reversible and evaluated easily, thus providing a promising substrate for the development of biomarkers for the detection and prevention of cancer, including colorectal cancer (CRC). In the present study, the protein expression and methylation status of smooth muscle protein 22α (SM22α) was investigated in 78 cases of CRC and adjacent normal tissue. The aim of the study was to investigate the function of SM22α in the pathogenesis of CRC and to identify a candidate biomarker for the early detection of CRC. The methylation status of promoter of SM22α gene was detected using methylation-specific polymerase chain reaction. The protein expression of SM22α was evaluated using western blot analysis. The results demonstrated a significant decrease of SM22α protein expression in 50 (68.5%) cases of CRC compared with that in adjacent normal tissues (P<0.001). The methylation status of SM22α promoter in CRC was significantly increased compared with that in adjacent normal tissues (P<0.001). Additionally, there was a negative correlation between the expression of SM22α protein and methylation levels of SM22α gene in CRC (P<0.001). Kaplan-Meier curves revealed that patients with CRC with an unmethylated promoter of SM22α gene exhibited an increased survival time (34.8±0.6 vs. 30.9±1.3 months; P=0.025) compared with that in patients with a methylated promoter of SM22α gene. The present study demonstrated that the protein expression of SM22α is downregulated in CRC tissues by hypermethylation of its promoter, and that the methylation of SM22 α promoter may be used as a biomarker for early detection, prognosis and prediction of CRC.

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Introduction

Colorectal cancer (CRC) is a common gastrointestinal malignancy. It is the third most common cancer worldwide and accounts for >600,000 mortalities annually (1,2). The 5-year relative survival rate for patients with CRC is <65% (3). Early diagnosis and treatment are crucial to increase the survival rate of patients with CRC. However, there is a lack of highly sensitive and specific biomarkers for detection and prognosis of CRC.

CRC is a complex disease that is caused by genetic and epigenetic alterations (4,5). Epigenetic alterations are stable changes in gene expression, without altering the underlying DNA sequence (6). DNA methylation is a common epigenetic alteration and serves a key function in the regulation of gene activity. Hypermethylation in gene promoter regions may lead to transcriptional silencing (7). Silencing of tumor suppressor genes is a crucial mechanism involved in carcinogenesis (8). Gene methylation may be reversible and may be modified by environmental factors (9). It is reasonable to hypothesize that methylated genes may be attractive candidates for the detection and prevention of cancer. Previous studies suggested several aberrantly methylated tumor suppressor genes to be used as biomarkers for the early detection and prognosis of CRC (10,11). However, their diagnostic sensitivity and specificity remains unsatisfactory.

Smooth muscle protein 22α (SM22α), also known as transgelin or TAGLN, is an actin-binding protein that is abundantly expressed in smooth muscle cells in vertebrates (12). Although the biological function of SM22α remains unclear, it has been suggested to regulate muscle fiber contractility, cell differentiation, cell migration, tissue invasion and tumor suppression (13-16). Accumulating evidence suggests that SM22α may act as a tumor suppressor. Previous studies reported decreased expression of SM22α in several types of human cancer, including lung, prostate, renal and breast cancer (17-20). The function of SM22α was also reported to be associated with increased apoptosis of prostate cancer cells (21). Furthermore, it was demonstrated that SM22α may suppress the expression of matrix metalloproteinase 9 (MMP-9), which serves an important function in tumor progression (22). A previous study demonstrated that SM22α expression was significantly decreased in CRC (23). However, the molecular mechanism underlying the downregulation of SM22α in CRC remains to be elucidated.

In the present study, the methylation status and protein expression of SM22α is examined in CRC and adjacent normal tissue. To the best of our knowledge, this is the first study to investigate the association between the protein expression and methylation level of SM22α in CRC tissues and their adjacent normal tissues. The aim of the present study is to investigate the function of SM22α in the pathogenesis of CRC, and to identify candidate biomarkers for the detection and prognosis of CRC.

Materials and methods

Tissue extraction. A total of 78 CRC tissues and adjacent normal tissues were obtained from the Department of General Surgery of the Fourth Hospital of Hebei Medical University (Hebei, China) between October 2013 and November 2014. The mean age of the patients was 62 years (range, 42-78 years). A total of 45 cases were male and 33 were female. Postoperative pathological examination confirmed the diagnosis of CRC. No patients received chemotherapy, radiotherapy or immunotherapy prior to surgery. Adjacent normal tissues were collected ≥10 cm away from the edge of the tumor. CRC tissues and corresponding adjacent normal tissues were snap frozen in liquid nitrogen within 30 min after their removal and stored at -80˚C. Following surgery, the patients were followed-up every 3 months. Overall survival was defined as the time from the date of surgery to the date of mortality due to CRC. Disease-free survival was defined as the time from the primary surgical treatment to the date of tumor recurrence or the last follow-up. At the time of the last follow-up, 18 (23.08%) had succumbed to disease, 7 (8.97%) were alive with disease and 53 (67.95%) were alive without disease. The present study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (Hebei, China) and written informed consent was obtained from all individuals.

Western blot analysis. Total protein was extracted from tissues using a lysis buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% sodium deoxycholate and 1 mM phenylmethanesulfonyl fluoride. Total protein was quantified using the Lowry method. Equal amount of protein (30 μg) was separated by SDS-PAGE (10% gels) and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) at room temperature for 2 h, and then incubated with rabbit polyclonal antibodies against SM22α (dilution, 1:1,000; Abcam, Cambridge, UK) and GAPDH (dilution, 1:800; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. Membranes were washed four times with TBST. Following primary incubation, were incubated with Anti-Rabbit IgG (H&L) (Goat) Antibody IRDye® 800CW conjugated (dilution, 1:20,000; cat. no. 611-131-122; Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) for 1 h at room temperature. The immunoreactive bands were visualized using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). For quantification, the bands were analyzed using ImageQuant software (version 3.0; LI-COR Biosciences), and the signal densities of the SM22α bands were normalized to those of the GAPDH bands. SM22α expression was quantified as a ratio to GAPDH expression (SM22α/GAPDH ratio). The experiment was repeated three times.

DNA extraction and bisulfite modification. Total DNA was extracted from tissues using the TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). The purity and concentration of the DNA was evaluated using a UV spectrophotometer. DNA (500 ng) was modified by sodium bisulfite using the EZ DNA Methylation-Gold kit (Zymo Research Corp., Irvine, CA, USA), according to the manufacturer's protocol.

Methylation-specific polymerase chain reaction (PCR) (MSP). Methylation-specific PCR primers were designed using MethPrimer software (Sangon Biotech Co., Ltd., Shanghai, China). The primers for methylated SM22α were as follows: 5'-AATAGTGAAGTAGGAGTAGTCGTA AGTTC-3' (forward) and 5'-AATCTACCGAAACTACCG AAAC-3' (reverse). The primers for unmethylated SM22α were as follows: 5'-GAATAGTGAAGTAGGAGTAGTTGT AAGTTT-3' (forward) and 5'-CAATCTACCAAAACTACC AAAAC-3' (reverse). The PCR reaction contained Platinum SYBR-Green qPCR SuperMix-UDG (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (12.5 μl), template DNA (1 μl), primers (each 0.5 μl) and diethylpyrocarbonate H_2O (10.5 μl). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 40 cycles at 95˚C for 30 sec, annealing at 64˚C for 30 sec, elongation at 72˚C for 45 sec and extension at 72˚C for 10 min. The PCR product for methylated and unmethylated SM22α was 149 and 151 bp, respectively. Amplification products were analyzed by 2% agarose gel electrophoresis. Peripheral blood DNA that was treated with SssI methyltransferase (New England BioLabs, Inc., Ipswich, MA, USA) was used as a positive control and deionized water was used as a negative control. The gel was visualized under UV illumination.

Statistical analysis. Data were analyzed using SPSS software (version 21.0; IBM Corp., Armonk, NY, USA). Quantitative values of protein expressions in CRC tissues and adjacent normal tissues were analyzed with paired Student's t-test and expressed as mean ± standard deviation (SD). The methylation rate of SM22α between CRC tissues and adjacent normal tissues were analyzed using the χ^2 test. The Association of the protein expression and methylation status of SM22α with clinical parameters of patients with CRC was compared using the χ^2 test. The log-rank test and Kaplan-Meier survival curve method were used for survival analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protein expression and methylation level of SM22α in CRC tissues and adjacent normal tissues. The relative protein expression of SM22α was analyzed in 78 pairs of CRC tissues and their adjacent normal tissues using western blot analysis (Fig. 1). The results demonstrated a significantly decreased expression of SM22α in 50 (68.5%) cases of CRC. Additionally,

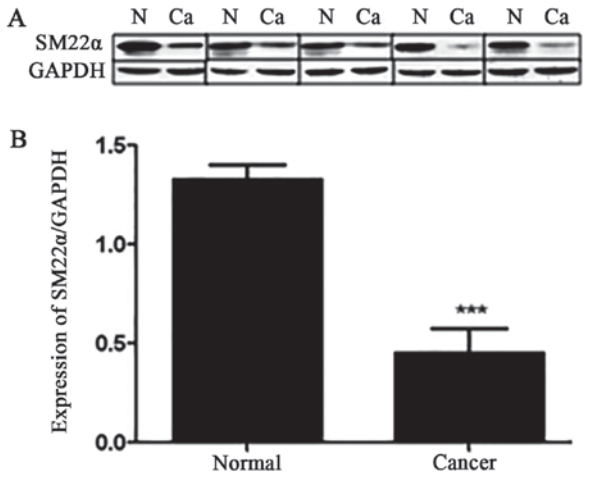

Figure 1. Expression of SM22α in colorectal cancer tissues and adjacent normal tissues. (A) Representative images and (B) quantification from western blot analysis of SM22α expression in 78 colorectal cancer tissues and their adjacent normal tissues. ***P<0.001 vs. normal tissues. N, adjacent normal tissues; Ca, colorectal cancer tissues; SM22α, smooth muscle protein 22α.

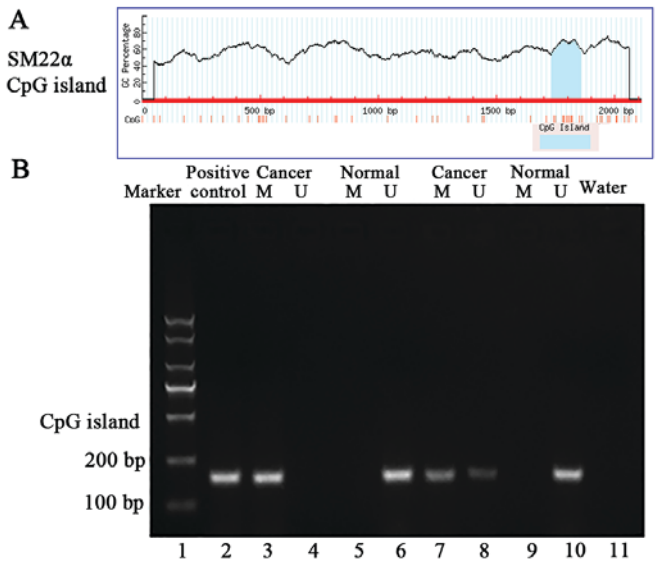

Figure 2. MSP of the CpG island of SM22α in colorectal cancer and adjacent normal tissues. (A) UCSC genome browser view of SM22α and distribution of CpG sites. (B) MSP products for SM22α promoter regions on an agarose gel. Lane 1, DNA marker; lane 2, methylation-positive control; lane 3-6, a representative case of colorectal cancer; lane 7-10, a representative case of colorectal cancer; lane 11, negative control. M, methylated; U, unmethylated; SM22α, smooth muscle protein 22α; MSP, methylation-specific polymerase chain reaction.

28 (31.5%) cases of CRC exhibited an unchanged or upregulated expression of SM22α. Fig. 1A shows representative western blot analysis of the expression of SM22α from five cases of CRC. SM22α was decreased by 2-fold in CRC tissues compared with that in adjacent normal tissues (mean ± SD, 0.7280±0.1412 vs. 1.4458±0.3433; paired Student's t-test, P<0.001; Fig. 1B).

To investigate the molecular mechanisms of SM22α gene regulation in CRC, DNA methylation levels within promoter CpG islands, were evaluated. The results demonstrated that methylation of SM22α CpG island in CRC tissues and adjacent normal tissues was 59.0% (43/78) and 21.9% (19/78), respectively. The difference was statistically significant (χ^2=15.418, P<0.001; Fig. 2) (Data not shown). Fig. 2B shows data from two cases of CRC.

Association of the protein expression and methylation status of SM22α with clinical parameters of patients with CRC. Statistical analysis of SM22α protein expression, SM22α gene methylation and clinical variables was performed using the χ^2 test. The expression of SM22α protein and methylation levels of SM22α gene were not associated with age, sex, tumor differentiation, tumor stage, lymph node metastasis, tumor infiltration depth or tumor location in patients with colorectal cancer (Table I).

Association of the protein expression and methylation status of SM22α in CRC tissues. Patients with CRC were divided into SM22α high- and low-expression groups on the basis of western blot analysis. The association between the protein expression and the methylation status of SM22α was determined in CRC tissues. SM22α was methylated in 40 of 43 cases of CRC with low protein expression of SM22α. SM22α was unmethylated in 25 of 35 cases of CRC with increased protein expression of SM22α. Therefore, there was a negative association between the protein expression and methylation levels of SM22α in CRC (P<0.001; Table II).

Methylation status of SM22α and survival time in patients with CRC. Kaplan-Meier survival curves revealed that patients with CRC with unmethylated promoter of SM22α presented a significantly longer overall survival time (34.8±0.6 months) compared with that in patients with a methylated SM22α promoter status (30.9±1.3 months; log-rank test, P=0.025; Fig. 3A). Additionally, patients with CRC with unmethylated promoter of SM22α exhibited a longer disease-free survival time (32.5±1.3 months) compared with that in patients with a methylated SM22α promoter status (26.0±1.9 months; log-rank test, P=0.027; Fig. 3B).

Discussion

SM22α is an early differentiation marker of smooth muscle cells, which is expressed in fibroblasts and epithelial cells (13,14). SM22α serves an important function in stabilizing the cellular structure and maintaining the differentiated phenotype of smooth muscle cells via association with actin (24,25). Previous studies have demonstrated that SM22α may be involved in the development and progression of malignant tumors. Decreased expression of SM22α has been reported in lung, prostate, renal and breast cancer (17-20). Zhang *et al* (21) reported that SM22α may induce apoptosis via interacting with p53 in prostate cancer cells. Nair *et al* (22) demonstrated that SM22α repressed the expression of MMP-9 via reducing the transactivation of activating protein 1-dependent and compromising the activation of extracellular signal-regulated kinase. Li *et al* (26) demonstrated that SM22α may decrease proliferation and invasion, and increase apoptosis in colorectal carcinoma cells. Xu *et al* (27) reported that SM22α may prevent the metastasis of CRC. These

Table I. Association between protein expression and methylation level of SM22α and clinicopathological characteristics.

		Decrease of SM22α protein expression		SM22α methylation	
Clinical parameters	Number (n)	Cases (n)	P-value[a]	M (n)	P-value[a]
Age (years)					
<60	31	22	0.305	19	0.374
≥60	47	28		24	
Sex					
Male	45	26	0.174	23	0.405
Female	33	24		20	
Differentiation					
Level I-II	62	41	0.463	35	0.644
Level III	16	9		8	
TNM stage					
I-II	49	29	0.239	25	0.343
III	29	21		18	
Lymphatic metastasis					
No	51	30	0.182	28	0.956
Yes	27	20		15	
Infiltration depth					
T1-T2	21	13	0.806	9	0.186
T3-T4	57	37		34	
Tumor location					
Colon	29	19	0.841	16	0.995
Rectal	49	31		27	

[a]Statistical analysis was performed using the χ^2 test. TNM, Tumor-Node-Metastasis; SM22α, smooth muscle protein 22α; M, methylation.

Table II. Association between protein expression and methylation level of SM22α in colorectal cancer tissues.

SM22α promoter methylation	SM22α protein expression			
	High[a]	Low[b]	χ^2	P-value
Methylated	3	40		
Unmethylated	25	10	34.832	<0.001

[a]SM22α protein expression was increased in colorectal cancer tissues compared with that in adjacent normal tissues. [b]SM22α protein expression was decreased in colorectal cancer tissues compared with that in adjacent normal tissues. SM22α, smooth muscle protein 22α.

findings indicate that SM22α may act as a tumor suppressor. However, the pathological function of SM22α may depend on the type of cancer. For example, upregulation of SM22α has been reported in gastric cancer and esophageal squamous cell carcinoma (28,29). Therefore, further research on the expression and function of SM22α in various tumor types is required.

The results of the present study demonstrated a decreased expression of SM22α in CRC tissues compared with that in adjacent normal tissues. However, the protein level of SM22α was not associated with age, sex, tumor differentiation, tumor stage, lymph node metastasis, tumor infiltration depth or tumor location. This implies that SM22α may be used as a biomarker of colorectal carcinogenesis and may serve as a tumor suppressor. These results are consistent with those of previous studies (23,30,31). However, Zhou *et al* (32) and Lin *et al* (33) reported that elevated levels of SM22α increased invasiveness and lymph node metastasis in CRC. The discrepancy between these results and the present findings may arise from the limited sample size.

The expression of SM22α is regulated at the transcriptional level (34). Yamamura *et al* (35) demonstrated that the transcriptional activity of SM22α was regulated by the methylation of the promoter region in smooth muscle cells. Zhao *et al* (30) revealed that treatment with 5-aza-2-deoxycytidine may restore the mRNA and protein levels of SM22α in the human intestinal epithelial cell line HT29. However, the association between the protein expression and methylation status of SM22α in CRC tissues and adjacent normal tissues remains unclear. The results of the present study revealed an increased methylation level of SM22α in CRC tissues compared with that in adjacent normal tissues. Additionally, there was a negative association between the methylation level and protein expression of

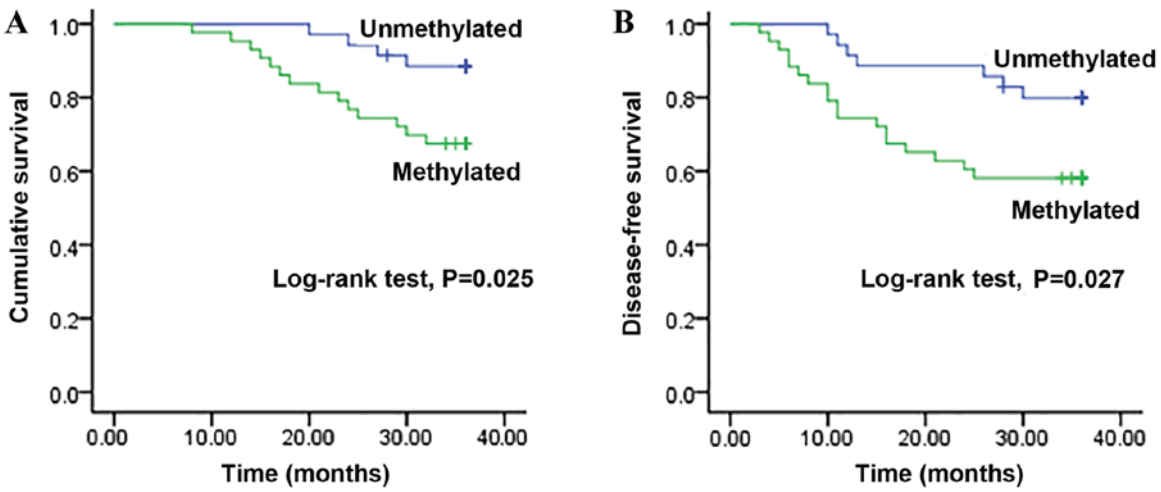

Figure 3. Kaplan-Meier survival analysis for patients with colorectal cancer with methylated and unmethylated levels of SM22α. (A) Overall survival and (B) disease-free survival of patients with colorectal cancer with unmethylated or methylated promoter of SM22α. SM22α, smooth muscle protein 22α.

SM22α in CRC tissues. The results indicated that hypermethylation of SM22α may be important in the regulation of SM22α transcription.

DNA hypermethylation results in gene silencing, and silencing of tumor suppressor genes is involved in carcinogenesis (8,36). Aberrantly methylated tumor suppressor genes may act as potential biomarkers for the early detection of tumors. The results of the present study demonstrated that the methylation level of SM22α promoter was increased in CRC tissues compared with that in adjacent normal tissues. However, methylation of SM22α promoter was not associated with age, sex, tumor differentiation, tumor stage, lymph node metastasis, tumor infiltration depth or tumor location. These results suggested that hypermethylation of SM22α gene may occur at early stages of colorectal carcinogenesis and therefore may be a biomarker for the early diagnosis of CRC. Additionally, patients with an unmethylated promoter of SM22α gene exhibited a longer survival time compared with that in patients with methylated promoter of SM22α gene, indicating that the methylation status of SM22α promoter region may be associated with the prognosis of patients with CRC.

The present study has several limitations. First, the sample size was small and further large-scale studies are required to confirm the results of the present study. Secondly, methylation status and protein expression of SM22α was not evaluated in healthy individuals. Finally, further studies are required to evaluate the methylation levels of SM22α in the plasma or serum in patients with CRC.

In conclusion, the results of the present study demonstrated that the protein expression of SM22α was significantly decreased in patients with CRC. Additionally, the methylation level in the SM22α gene promoter region was increased in CRC tissues compared with that in adjacent normal tissues. There was a negative association between the protein expression and methylation levels of SM22α. Kaplan-Meier survival analysis revealed that patients with CRC with an unmethylated promoter of SM22α gene exhibited an improved survival time compared with that in patients with methylated promoter of SM22α gene. Therefore, the methylation level of SM22α promoter may be a useful biomarker for early detection, prognosis and prediction of CRC.

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Authors' contributions

BL, YL and EW designed the study and applied for approval from the Research Ethics Board. YL and DK recruited the patients and collected the data. BL, YL and JZ analyzed the data and prepared draft figures and tables. YL prepared the manuscript draft with important intellectual input from BL, EW and JZ.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (Hebei, China) and written informed consent was obtained from all individuals.

Consent for publication

All study participants provided written informed consent for the data to be published.

Competing interests

The authors declare that they have no competing interests.

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