High methylation of the *SEPT9* gene in Chinese colorectal cancer patients

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ABSTRACT. Methylation of the septin 9 gene (*SEPT9*) occurs in higher frequency in colorectal cancer (CRC) compared to control samples, which suggests that *SEPT9* methylation is a useful biomarker for screening CRC. However, the methylation status of *SEPT9* in Chinese CRC patients is scarcely reported. In the present study, *SEPT9* methylation was tested in CRC tissues obtained from a Chinese population and correlations with pathological characteristics were investigated. The methylation status of *SEPT9* was detected using methylation-specific polymerase chain reaction (PCR)-denaturing high-performance liquid chromatography (MSP-DHPLC) in 234 colorectal tissues (172 cases, 62 controls). Samples were sequenced to confirm the results from MSP-DHPLC. The chi-squared test was used to analyze the correlation of *SEPT9* gene methylation status and pathological characteristics in CRCs. *SEPT9* gene methylation was detected in 152 of 172 (88.4%) cases of verified CRC and in 4 of 62 (6.5%) healthy controls ($\chi^2 = 137.62$, P < 0.001). There was no association between the methylation status of *SEPT9* and age, gender,

Duke's stage, TNM stage, differentiation, and site of cancer (P > 0.05). Our results suggest that *SEPT9* gene methylation is a valuable biomarker for screening CRC in the Chinese population.

Key words: *SEPT9*; Methylation; Colorectal cancer; Chinese population

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignant tumor in males and the second most common in females worldwide. There were over 1.2 million new cancer cases and 608,700 estimated cancer deaths reported in 2008 (Jemal et al., 2011). The CRC incidence rates are rapidly increasing in a number of countries within Eastern Asia, including Japan and China (Center et al., 2009). The survival rate of colon cancer has increased over the past 20 years owing to early detection from increased screening and improved treatment. The fecal occult blood test (FOBT), flexible sigmoscope, and colonoscopy are common methods to screen early CRC (Cunningham et al., 2010). It is estimated that CRC deaths could be reduced by about 60% if patients are screened routinely (He and Efron, 2011). With the development of molecular biology and the availability of molecular markers, great progress has been made in screening CRC (Baylin et al., 2001; Creeden et al., 2011; Lind et al., 2011). The methylation of the septin (SEPT) 9 gene (*SEPT9*) is one of the molecular markers commonly used in detecting CRC (Feinberg, 2004; Lofton-Day et al., 2008; Warren et al., 2011).

Septins belong to a GTP binding protein family and currently consists of 14 members in mammals (Bourne et al., 1991; Leipe et al., 2002; Connolly et al., 2011). The association of septins with actin and tubulin are required in cytokinesis (Spiliotis et al., 2005; Kinoshita, 2006). Due to their essential role in cell division and polarity, it is not surprising that septins were also reported to be key players in tumorigenesis (Osaka et al., 1999; Garcia-Fernandez et al., 2010; Jia et al., 2010). *SEPT9* has been found to act as an oncogene and tumor suppressor gene in different types of cancers. Moreover, *SEPT9* was reported to fuse to the mix lineage leukemia (MLL) in leukemia, providing further evidence to support its oncogenic role (Saito et al., 2010; Santos et al., 2010). In hormone-regulated cancers, such as prostate, ovarian, and breast cancer, overexpression of *SEPT9* is accompanied by upregulation of hypoxia-inducible factor 1α (HIF-1α) (Zhong et al., 1999; Scott et al., 2005, 2006; Amir et al., 2009), which is probably regulated by estrogen receptor (ER) signaling (Amir et al., 2009). Loss of expression of *SEPT9* has been reported in head and neck cancer (Bennett et al., 2008) and CRC (Grutzmann et al., 2008).

Studies have shown that *SEPT9* methylation occurs in CRC and its detection from peripheral blood is feasible, whereas the frequency of *SEPT9* methylation in control samples is low (deVos et al., 2009; Tänzer et al., 2010). This suggests that *SEPT9* methylation is a useful biomarker for screening CRC. Consistent with gene methylation, the expression of SEPT9 was significantly reduced in CRC patients compared to healthy controls. The mRNA and protein levels of *SEPT9* increased in HT29 cells after demethylation treatment (Tóth et al., 2011). Colorectal tumorigenesis is highly correlated with environmental factors (Hall and Crowe, 2011); therefore, the *SEPT9* methylation status of CRC in the Chinese population may be different from that of other populations. However, studies on the *SEPT9* methylation of CRC in the Chinese population are scarce (He et al., 2010), and the relationship between *SEPT9* methylation and gender, differentiation level, and stage remains unclear. In the present

study, we detected the methylation status of *SEPT9* using methylation-specific polymerase chain reaction (PCR)-denaturing high-performance liquid chromatography (MSP-DHPLC) in CRC patients and healthy controls in Inner Mongolia, China. The association between CRC pathological characteristics and methylation of *SEPT9* was also analyzed.

MATERIAL AND METHODS

Subjects

In total, 234 subjects were enrolled from the Affiliated Hospital of Inner Mongolia Medical College, including 172 colorectal cancer patients (106 males, 66 females; mean age 61.29 ± 11.40 years) and 62 healthy controls (26 males, 36 females; mean age 53.00 ± 13.77 years). Paraffin-embedded CRC tissues and peripheral blood from healthy controls were used in this study. Pathological diagnosis confirmed that cancer cells occupied >70% in the CRC tissues used in this study. The clinicopathological characteristics of patients are shown in Table 1. Standard informed consent was obtained from all participating subjects according to the protocol reviewed and approved by the Ethical Committee of the Affiliated Hospital of Inner Mongolia Medical College.

Table 1. Clinicopathologic characteristics of patients and the correlation between the methylation status of *SEPT9* gene and pathological characteristics in colorectal cancer tissue.

Groups	Cases	SEPT9		χ^2	P value
		Methylation	Unmethylation		
Gender					
Male	106	95	11	1.442	0.230
Female	66	55	11		
Age (year)					
<50	26	20	6	5.169	0.160
50-59	38	36	2		
60-69	50	42	8		
>70	58	52	6		
Differentiation					
Moderate	152	133	19	0.099	0.753
Well	20	17	3		
Site					
Rectum	102	90	12	0.237	0.627
Colon	70	60	10		
Dukes' stage					
A	87	78	9	0.944	0.331
B	85	72	13		
TNM stage					
T1N0M0	62	55	7	1.274	0.529
T2N0M0	26	24	2		
T3N0M0	84	71	13		

DNA extraction and modification

Ten pieces of 5 μm paraffin sections were cut from each sample. The paraffin sections were then added into Eppendorf tubes with xylene, and were washed with ethanol three times. DNA was extracted using the QIAamp® DNA FFPE Tissue Kit (Qiagen, Germany). The purity and concentration of DNA were analyzed with agarose gel electrophoresis and a UV spectro-

photometer. The Whole Blood Genomic DNA Extraction Kit (TIANGEN, Beijing, China) was used to extract genomic DNA from the peripheral blood of healthy controls. DNA with treatment of enzyme Sss was used as the positive control, whereas DNA without treatment of enzyme Sss was used as the negative control. Sulfurous acid salt treatment was performed using the EZ DNA Methylation Gold Kit (Zymo Research, CA, USA). DNA was eluted in 30 μL elution buffer.

Amplification of *SEPT9*

Methylation-specific PCR primers were designed according to the GenBank NG_011683 sequence. The *SEPT9*-M primers were forward 5'-TCCGAAATAATCCAACT-3' and reverse 5'-CGTAGGGTTCGGGTTTCGT-3', and the *SEPT9*-U primers were forward 5'-GTGTAGTTGGATGGGATTATT-3' and reverse 5'-CCATCTCCCCTCAACACACTCCCA-3'. HotStarTaq DNA polymerase (QIAGEN) was used in DNA amplification. A touchdown PCR protocol was used for amplification of *SEPT9* under the following program: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. DNA from peripheral blood lymphocytes (PBL) of the healthy controls with treatment of enzyme Sss were used as methylated positive controls. Distilled water was used as the template for the negative control. PCR products (150 bp) were separated by 2% agarose gel electrophoresis and were stained with ethidium bromide. The gel was photographed using the UV Gel Documentation System.

DHPLC analysis

The amplification products were analyzed using DHPLC with the following steps: equal amounts amplification products of *SEPT9*-M PCR and *SEPT9*-U PCR were mixed and 5 μL mixture was used for further analysis. The PCR products were separated by a DNASep® analytical column (Transgenomic, Inc.) and were analyzed by DHPLC on the WAVE™ DNA Fragment Analysis System. The analytical procedure was DS Multiple Fragments and the 100-150-bp fragments were analyzed at 50°C at a 0.9 mL/min flow rate, and were detected by UV wave at 260 nm. The results were analyzed using the Navigator software.

Sequencing

To confirm the gene methylation status from the DHPLC analysis, we performed sequencing in randomly selected samples. The primers for sequencing were *SEPT9*-M forward 5'-TATTAGTTATTATGTCGGATTTCGC-3' and reverse 5'-GCCTAAATTAAAAATCCCGTC-3', and *SEPT9*-U forward 5'-ATTAGTTATTATGTTGGATTTTGTGG-3' and reverse 5'-AAAACACCTAAATTAAAAATCCCATC-3'. DNA sequences were verified by direct sequencing (ABI Prism 3700 DNA analyzer 377; Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis was performed with the SPSS 13.0 software. Pathological characteristics and differences in *SEPT9* methylation between the CRC patients and controls were compared using the Pearson chi-squared test.

RESULTS

The sensitivity of detecting the methylated *SEPT9* gene was analyzed by MSP-DH-PLC to identify the minimum detectable amount of cancer cells. Positive controls and negative controls were mixed proportionally. The contents of positive controls were 50, 25, 10, and 1%. The sensitivity was determined to be at least 1% (Figure 1A). Subsequently, we detected the methylation statuses of *SEPT9* in all samples using MSP-DHPLC (Figure 1B). Samples showing a peak at the same time as the positive control in DHPLC were defined as positive, and were otherwise defined as negative.
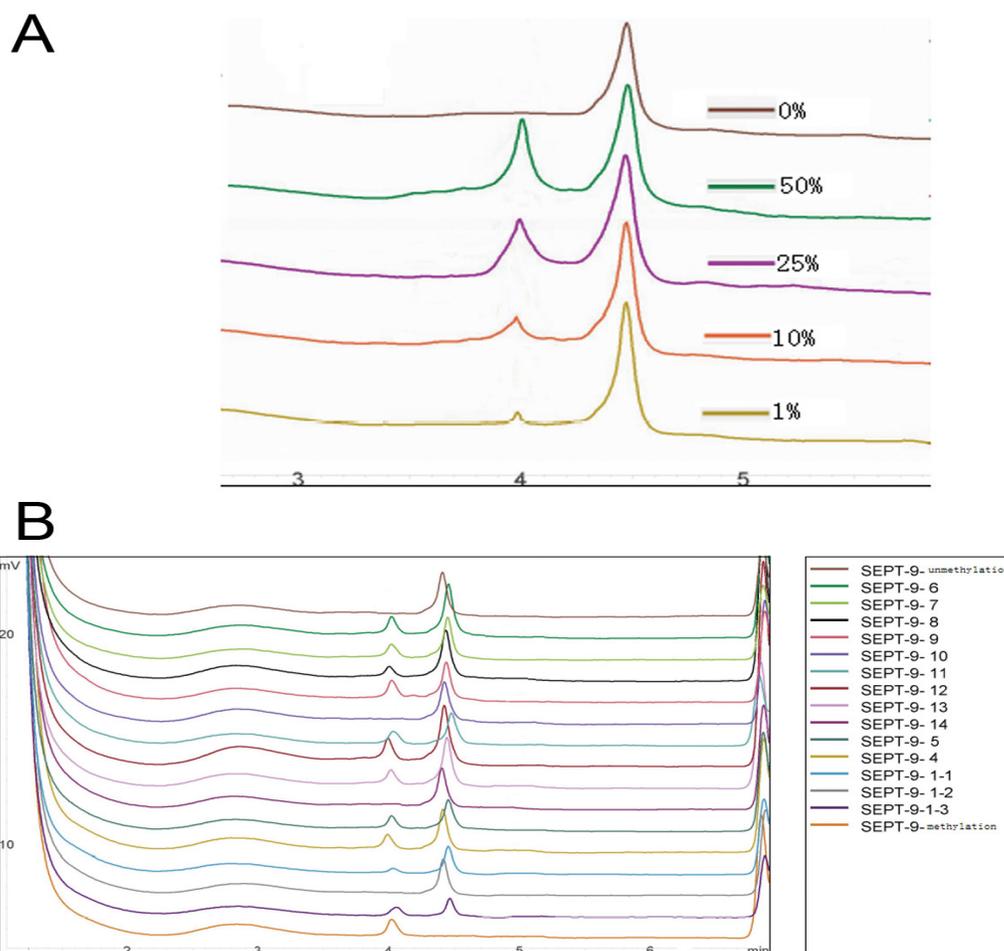
Figure 1. A. Assessment of sensitivity of MSP-DHPLC. Different concentrations (50, 25, 10, and 1%) of positive controls and negative control (0%, unmethylation) were used. **B.** Image of methylated *SEPT9* gene detected using MSP-DHPLC in some samples. SEPT-9-unmethylation = unmethylated control. SEPT-9-methylation = methylated control. SEPT-9-number = number of samples involved in this study.

Methylation of *SEPT9* was identified in 152 out of 172 (88.4%) CRC samples, whereas it was identified in only 4 out of 62 (6.5%) control samples. There was a significant difference in the methylation status of *SEPT9* between cases and controls ($\chi^2 = 137.62$, P < 0.001; Table 2).

Table 2. Distribution of methylation of *SEPT9* gene in CRC patients and controls.

Groups	SEPT 9		χ^2	P value
	Methylation	Unmethylation		
Cases	152 (88.4)	20 (11.6%)	137.62	<0.001
Controls	4 (6.5%)	58 (93.5%)		

Furthermore, we analyzed the correlation between the methylation status of *SEPT9* and gender, age, Duke's stage, TNM stage, differentiation, and site using the chi-squared test. There was no significant difference between any of the groups (P > 0.05, Table 1). To confirm the results of the methylation status of *SEPT9* from the DHPLC analysis, we sequenced the MSP-PCR products from randomly selected samples. The sequencing results were consistent with the results from DHPLC analysis.

DISCUSSION

MSP-DHPLC is a new assay that is currently used in clinics for the detection of mutations and methylations in China. The test is easy to perform, with high sensitivity and specificity (Divine et al., 2006). In our study, the frequency of *SEPT9* methylation was significantly different between CRC cases and controls (88.4 *vs* 6.5%, $\chi^2 = 137.62$, P < 0.001).

The proportion of methylated *SEPT 9* genes in CRC patients is 70-80%. The sensitivity and specificity of *SEPT9* methylation for detecting CRC in clinical samples is 88.4 and 93.5%, respectively. Therefore, the methylation of *SEPT9* is considered to be a specific biomarker of CRC (Grutzmann et al., 2008; deVos et al., 2009; Warren et al., 2011). The proportion of methylated *SEPT 9* genes in our CRC patients was higher (88.4%) than that observed in previous studies (deVos et al., 2009; Tänzer et al., 2010), and is likely correlated with the pathological diagnosis of patients. Furthermore, MSP with at least 1% detection sensitization was employed in this study. Whether the MSP method is correlated with experimental results is worth further investigation.

In the present study, we performed a correlation analysis of the methylation of *SEPT9* and pathological characteristics of CRC samples in Inner Mongolia of China. Our results showed that the methylation status of *SEPT9* was not associated with age, gender, Duke's stage, TNM stage, differentiation, or site. It was reported that the proportion of methylated *SEPT9* genes increased with the progression of CRC (deVos et al., 2009; Warren et al., 2011), whereas in our study, the proportion of methylated *SEPT9* was similar between well (17/20, 85%) and moderately differentiated (133/152, 87.5%) CRC cases. However, this result might be biased by the larger number of moderately differentiated CRC cases in this study. Furthermore, there was no metastasis observed in the CRC samples (Duke's stage and TNM stage from Table 1); therefore, whether methylation of *SEPT9* is involved in the metastasis of CRC needs further study in larger samples including cases of advanced CRC.

The Inner Mongolia region belongs to the alpine region where beef and mutton have served as the staple food with relatively less vegetables and fruits, and alcohol consumption is

customary of local residents. Further investigation is needed to determine the influence of environment and lifestyle on CRC. Moreover, the size of the sample of the present study is quite small; therefore, increasing the sample size is required to confirm these results in future studies.

In conclusion, our results indicated that the methylation status of *SEPT9* is a reliable index for screening CRC. Furthermore, the methylation of *SEPT9* detected by MSP-DHPLC is also a useful biomarker in a clinical laboratory setting. Meanwhile, the development of *SEPT9* methylation-specific drugs might be a new therapeutic approach for CRC.

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