Original Article

Detection and Clinical Significance of *DLC1* Gene Methylation in Serum DNA from Colorectal Cancer Patients

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ABSTRACT

Objective: *Deleted in liver cancer 1 (DLC1)* is a new candidate tumor suppressor gene, whose down-regulation or even silence will result from promoter hypermethylation in various human cancers including colorectal cancer (CRC). The aim of this study is to evaluate the diagnostic role of *DLC1* gene methylation in the serum DNA from CRC patients.

Methods: This study enrolled 85 CRC patients and 45 patients with benign colorectal diseases. Methylation-specific polymerase chain reaction (MSP) was used to determine the promoter methylation status of *DLC1* gene in serum DNA. The combination of *DLC1* methylation and conventional tumor markers was further analyzed.

Results: Hypermethylation of *DLC1* was detected in 42.4% (36/85) of CRC serums, while seldom in the benign controls (8.9%, 4/45) (P<0.001). The aberrant *DLC1* methylation in serum DNA was not associated with patients' clinicopathological features and elevated CEA/CA19-9 levels. Furthermore, the combinational analysis of CEA, CA19-9 and *DLC1* methylation showed a higher sensitivity and no reduced diagnostic specificity than CEA and CA19-9 combination for CRC diagnosis.

Conclusion: The serum *DLC1* methylation may be a promising biomarker for the early detection of CRC, which will further increase the diagnostic efficiency in combination with CEA and CA19-9.

Key words: *DLC1*; Colorectal cancer; Methylation; Biomarker

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in the world, with approximately one million new cases occurring per year. Although endoscopic screening has been widely used, most patients can only be diagnosed at the middle or advanced stages, thus the options for curative resections are limited and survival rate remains low[1]. It is clearly imperative to develop more efficient diagnostic methods to realize the early detection of cancer. Molecular markers would provide an alternative approach and among them, DNA methylation alterations in the promoter region of tumor suppressor genes (TSGs) show great promise[2]. Many studies have shown that TSGs were frequently silenced by promoter hypermethylation in CRC as well as pre-malignant lesions, indicating that the aberrant methylation as a molecular marker system could be used for early detection of cancer[3, 4]. Moreover, it has been demonstrated that methylated alteration also exists in "circulating DNA", such as DNA from blood, stool or ascitic fluid, making it well-suited for non-invasive detection[5-7].

Deleted in liver cancer-1 (DLC1) gene, first identified as a rat *p122RhoGAP* gene homolog in hepatocellular carcinoma (HCC)[7], is considered to be a new candidate TSG. It is located on chromosome 8p21.3–22, a region frequently deleted in tumors[8]. Ectopic expression of *DLC1* has been found to induce cell morphological changes or to suppress cell proliferation, migration, and colony formation in the cells of HCC, renal cancer, lung cancer and prostate cancer, etc[9-11]. In addition，promoter hypermethylation of *DLC1* has been found in various human cancers including CRC, linked with the absence of the *DLC1* gene expression[12, 13]. It has also been revealed that *DLC1* methylation status in non-Hodgkin's lymphoma can be used as a diagnostic marker[14]. However, very few attempts have been made to clarify the value of *DLC1* methylation in CRC diagnosis.

Therefore, this study tries to identify the methylation status of *DLC1* in the serum DNA from CRC patients. The combination of *DLC1* methylation and conventional tumor

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markers for CRC detection can efficiently increase the diagnostic rate.

MATERIALS AND METHODS

Study Population

This study enrolled 85 CRC patients diagnosed at Departments of General Surgery of Jiangsu Cancer Hospital and Affiliated Zhongda Hospital, Southeast University from April, 2008 to April, 2011. None had received preoperative chemotherapy or radiation therapy. All patients were diagnosed adenocarcinoma based on pathological and/or cytological evidence. Tumor stage was determined according to the Duke's staging system, and cellular differentiation was graded according to the Broders' grading system. The control population consisted of 45 patients with benign colorectal diseases (benign polyp, nonmalignant adenoma, ulcerative colitis, etc.). Ethical approval was obtained from the hospital and fully informed consent from all patients prior to sample collection. Peripheral blood samples of about 5 ml were collected from each patient by using tubes containing clot activator. After standing for 2 h, the serum was isolated by centrifuging at 3,000 r/min for 10 min and stored at –80°C until use.

Determination of Carcinoembryonic Antigen (CEA) and Carbohydrate Antigen 19-9 (CA19-9)

The concentrations of CEA and CA19-9 of part of the patients came from their routine biochemical examination on the next day after admission, which was determined by an automated immunoassay system (Elecsys 2010, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Serum levels of CEA greater than 5.0 ng/ml and CA19-9 greater than 37 U/ml were considered positive.

Methylation-specific Polymerase Chain Reaction (MSP) and Bisulphite Sequencing

Serum DNA was extracted by using QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. All of the final 50μl eluted DNA was bisulphite-treated with EZ-DNA methylation Gold Kit immediately (Zymo Research, Orange, CA, USA). The bisulphite-modified DNA was resuspended in 10 μl of TE buffer. Then methylation status of *DLC1* promoter was determined by MSP and the reaction system was performed in a 25 μl volume containing 5 μl of DNA template, 10×Buffer, 0.15 mmol/L dNTP, 0.1 mmol/L each primer and 0.5 U of Ex Taq Hot Start Version (Takara, Shiga, Japan). Primers used in the study have been listed in Table 1. Lymphocyte DNA, original or methylated *in vitro* by excessive CpG (SssI) methylase (New England Biolabs, Beverly, MA, USA), was used as unmethylation and methylation positive control. Water blank was used as a negative control. To verify the MSP results, a 292-bp fragment specific to the upstream region of the basic promoter of *DLC1* was amplified, and the PCR product was sequenced.

Table 1. List of all primers used and conditions of PCR amplification

Primer	Sequence(5'-3')	Product size	PCR condition
DLC1(M)	f: TTTAAAGATCGAAACGAGGGAGCG r: CCCAACGAAAAACCCGACTAACG	172 bp	94°C 30s, 52°C 30s, 72°C 30s, 45 cycles
DLC1	f: TTTTTTAAAGATTGAAATGAGGGAGTG r: AAACCCAACAAAAAAACCCAACTAACA	178 bp	94°C 30s, 52°C 30s, 72°C 30s, 45 cycles
DNA (PCR)	f: GTTTTTAGTTAGGATATGGT r: CTTCTTTCTACACATCAAACA	292 bp	94°C 30s, 52°C 30s, 72°C 30s, 45 cycles

M, methylation-specific primers; U, unmethylation-specific primers; f, forward; r, reverse

Statistical Analysis

Differences in frequency were assessed by Chi-square test or Fisher's exact test. Statistical analyses were performed using SPSS version 12.0 for windows (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

RESULTS

DLC1 Methylation Profile in Serum DNA of CRC Patients

Firstly the methylation status of *DLC1* in serum DNA was determined in cancer and benign control groups. The results showed that, hypermethylation of the *DLC1* promoter was detected in 42.4% (36/85) of CRC, while in control group was 8.8% (4/45), and the difference was all statistically significant ($P<0.001$). The representative agarose gel electrophoresis results are shown in Figure 1A. Attentions should be paid to some especial results, if the methylated and unmethylated bands were both detected in a sample, it was incomplete methylation (calculated as methylation); if the methylated and unmethylated bands were both not detected in a sample (calculated as unmethylation), it might be caused by the extremely low DNA content in serum.

To verify the MSP results, we performed high-resolution bisulphate genomic sequencing in the stochastic samples. In agreement with the results of the MSP, six CpG dinucleotides of *DLC1* gene in the sample #5 showed extensive hypermethylation, whereas sample #18 was unmethylated at these CpG dinucleotides (Figure 1B).

Clinicopathological Correlation of *DLC1* Methylation in Serum DNA of CRC

The relationship between *DLC1* promoter methylation status in CRC serum and patients' clinicopathological features was further analyzed. As showed in Table 2, *DLC1* methylation was not correlated with patients' sex, age, tumor site, differentiation grade, stage, lymph metastasis and distant metastasis. There was also no correlation of *DLC1* gene methylation status and serum levels of CEA and CA19-9.

Combination of *DLC1* Methylation and Conventional Tumor Markers

The overall detection rate of the *DLC1* methylation in CRC had no difference with the positive rate of CEA (38.8%, 33/85) and CA19-9 (34.1%, 29/85). However, the combinational analysis of CEA, CA19-9 and *DLC1* methylation showed a higher sensitivity than conventional combination of CEA and CA19-9 for CRC detection (70.6%, 60/85 vs. 52.9%, 45/85; *P*=0.018). Furthermore, the diagnostic specificity of tripartite combination was not reduced, compared with CEA and CA19-9 combination (60%, 27/45 vs. 66.7%, 30/45; *P*=0.512) (Table 3).

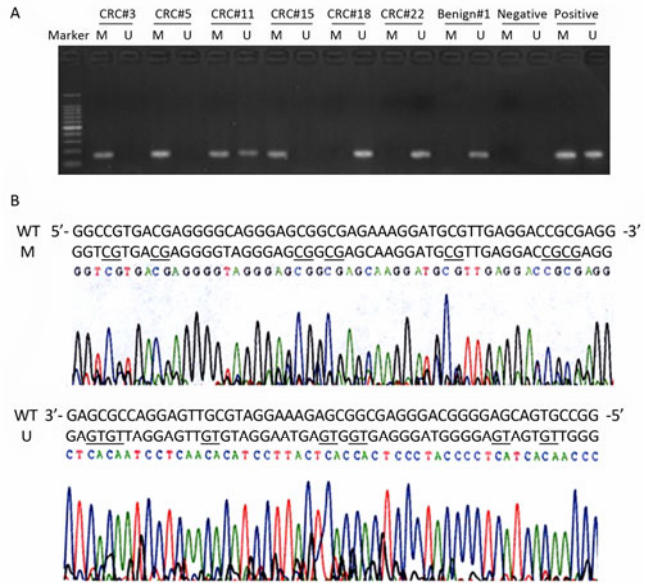

Figure 1. Methylation status of *DLC1* gene in serum DNA of CRC patients. A: Typical agarose gel electrophoresis of MSP results. CRC#3, #5 and #15 are examples of methylation (M), CRC#18, #22 and Benign#1 are examples of unmethylation (U), and CRC#11 is an example of incomplete methylation (analyzed as methylation). Lymphocyte DNA, original or methylated *in vitro* by excessive CpG (SssI) methylase was used as unmethylation and methylation positive control. Water blank was used as a negative control. B: Bisulfite sequencing of *DLC1* methylation, a 292-bp fragment of the *DLC-1* promoter region was sequenced. Examples of a highly methylated CpG island from the CRC#5 (M) and an unmethylated *DLC-1* 5'CpG island from the CRC#18 (U). Underlining indicates the positions of CpG dinucleotides.

Table 2. Association between the *DLC1* methylation in CRC serum and clinicopathological features (n)

Characteristics		No.	*DLC1* methylation		*P*
			M	U	
Age (year)	<60	45	17	28	0.365
	≥60	40	19	21	
Gender	Male	48	19	29	0.556
	Female	37	17	20	
Tumor site	Proximal	24	8	16	0.415
	Distal	25	13	12	
	Rectum	36	15	21	
Differentiation	Well/moderate	51	19	32	0.244
	Poor	34		17	
	Undifferentiation		17		
Stage	I/II	39	14	25	0.267
	III/IV	46	22	24	
Lymph Metastasis	No	40	14	26	0.196
	Yes	45	22	23	
Distant Metastasis	No	66	26	40	0.303
	Yes	19	10	9	
CEA	≥5.0 ng/ml	33	15	18	0.645
	<5.0 ng/ml	52	21	31	
CA19-9	≥37 U/ml	29	11	18	0.553
	<37 U/ml	56	25	31	

Table 3. Comparison of *DLC1* gene methylation rates and positive rates of tumor markers in serum

Patients	No.	*DLC1* methylation	CEA	CA19-9	CEA+CA19-9	*DLC1* methylation +CEA+CA19-9
CRC	85	36(42.4%)	33(38.8%)	29(34.1%)	45(52.9%)	60(70.6%)*
Controls[1]	45	4(8.8%)	10(22.2%)	8(17.8%)	15(33.3%)	18(40.0%)Δ

[1]Controls were 40 patients with benign colorectal diseases. *P<0.05, ΔP>0.05 (vs. CEA+CA19-9), Chi-square test

DISCUSSION

DNA methylation is an important regulatory mechanism of epigenetic modification. Hypermethylation will lead to the down-regulation or silence of TSGs and then contribute to carcinogenesis. Given the DNA methylation usually happens before genic changes, the abnormal methylation changes at CpG island of TSGs may be used as one of the available means for the early detection of tumor patients[15].

The new candidate tumor suppressor gene *DLC1* was isolated from human HCC by a PCR-based subtractive hybridization approach[7]. Determination of the *DLC1* cDNA sequence shows that it is the human homologue of rat p122, which has been found to act as a Rho GTPase activating protein (RhoGAP)[16]. Thus, the *DLC1* gene is considered to be a negative regulator of the Rho protein family of small GTPases and to control cytoskeletal rearrangement, membrane trafficking, gene expression, cell proliferation, malignant transformation, and metastatis[17,18]. Considerable evidence has accumulated to prove that *DLC1* acts as a bona fide TSG, for *DLC1* is widely expressed in many adult human tissues, but it is frequently downregulated by genomic deletions or DNA methylation in various human cancers, including HCC, breast cancer, ovarian cancer, uterine cancer, gastric cancer, lung cancer, pancreatic cancer, prostate cancer, renal cancer, nasopharyngeal cancer and colon cancer [12, 13, 19-21]. In addition, several studies show that reactivation of *DLC1* function results in suppression of tumor cell proliferation and induces caspase-3-mediated apoptosis *in vitro* as well as abolishes or reduces tumorigenicity *in vivo*[7, 8]. In CRC, our previous data suggest that *DLC1* gene is silenced by promoter methylation in HT29 cell line, and restoration of *DLC1* expression in HT29 cells inhibits cell proliferation and migration significantly, flow cytometry also shows *DLC1* transfected HT29 cells are induced apoptosis and cell cycle arrest [22]. Undoubtedly, *DLC1* methylation plays an important role in CRC development, this study currently investigates the diagnostic role of *DLC1* methylation in CRC.

It is well known that double-strand DNA fragments often appear in considerable quantities in the serum or plasma of cancer patients, which maybe arise from lysis of tumor cells[23]. Enriched DNA makes it possible to detect tumor-specific DNA alteration in the peripheral blood of patients. In the present study, we found a higher methylation frequency of *DLC1* gene promoter in the serum DNA from CRC patients, whereas it was rare in the serum of benign disease. The sensitivity, as well as the specificity was satisfactory compared with several other frequently methylated loci identified in plasma/serum of CRC, for example p15, p16, APC, hMLH1, MGMT, RASSF1A, RUNX3, SFRP1 and 2[24-26]. Additionally, consistent with Zhang, et al.[21], we did not find any correlation between *DLC1* methylation and clinicopathological features in CRC, which suggested *DLC1* methylation might be a relatively early event during tumorigenesis. Thus, *DLC1* methylation may be a new ideal serum biomarker for CRC early detection.

Conventional tumor markers have been widely used in clinical diagnosis. At present, considerable evidence reveals the advantages of DNA methylation over protein-based markers in many aspects, such as excellent specificity, relative stability and high sensitivity based on PCR approaches[27]. According to our data, the overall detection rate of the *DLC1* methylation in CRC patients was paralel to that of CEA and CA19-9. However, we found no correlation between *DLC1* methylation and elevated levels of CEA or CA19-9[28-30], which demonstrated that DNA methylation and conventional tumor markers could serve as complementary diagnosis markers. As was expected, the tripartite combination of CEA, CA19-9 and *DLC1* methylation showed a significantly higher sensitivity and no reduced diagnostic specificity than the bipartite combination of CEA and CA19-9. Thus, a combinational analysis of CEA, CA19-9 and *DLC1* methylation may be an intensive screening for CRC diagnosis. Meanwhile, other biomarkes are still under investigation for gastrointestinal cancers. This combination may be further improved with the occurance of other biomarkers.

In conclusion, our study showed *DLC1* gene promoter methylation has a higher detection rate in CRC serum. Its diagnostic value for CRC will be further confirmed with the increase of diagnostic efficiency when combined with CEA and CA19-9.

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