Detection of Aberrant p16 Methylation in the Serum of Colorectal Cancer Patients[1]

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ABSTRACT

Purpose: **This study was designed to detect aberrant p16 promoter methylation in the serum of patients with colorectal cancer (CRC) and to explore the possibility of using this assay in early detection or as a prognostic marker of CRC patients.**

Experimental Design: **Methylation-specific PCR was used to detect p16 methylation in DNA extracted from 52 CRCs and matching serum samples and control serum samples from 34 patients with adenomatous polyps and 10 healthy individuals. The association of p16 hypermethylation in serum DNA of CRC patients with clinicopathological characteristics was then analyzed.**

Results: **P16 hypermethylation was found in 20 of 52 (38%) CRCs. Among the 20 cases with aberrant methylation in the tumor tissues, similar changes were also detected in the serum of 14 (70%) cases. No methylated p16 sequences were detected in the peripheral serum of the other 32 CRC cases without these changes in the tumor, in 34 patients with adenomatous polyps, or in 10 healthy control subjects. Clinicopathological analysis revealed that p16 methylation in serum was significantly associated with later Dukes' stage ($P = 0.03$).**

Conclusions: **This assay offers a potential means for the serum-based detection and/or monitoring of CRC patients.**

INTRODUCTION

CRC[3] is one of the most common malignancies worldwide. Affected individuals (~40%) will ultimately die from cancer (1). The most effective treatment for CRC is surgical resection, but this modality is limited by the fact that nearly half of CRC patients have advanced disease at the time of diagnosis. The chance of cure is great in those individuals whose primary or recurrent tumors are detected at an early stage that permits curative surgery. One novel approach to cancer detection has been the attempt to detect tumor-specific DNA alteration in DNA extracted from blood of patients (2). We hypothesize that because aberrant promoter hypermethylation of tumor suppressor genes has been detected in the serum or plasma of patients with various cancers (1, 3–8), aberrant p16 methylation might be detectable in the peripheral blood of CRC patients. After a demonstration of aberrant methylation of the *p16* gene in CRC tissues, we attempted to detect the same methylation change in the serum of CRC patients using MSP.

MATERIALS AND METHODS

Sample Collection. Primary colon cancer samples and matching preoperative serum samples were collected from 52 patients who were operated at Ruijin Hospital of Shanghai Second Medical University (Shanghai, China) between 1999 and 2001. Histological confirmation of the diagnosis of CRC was obtained in all cases. Preoperative peripheral serum samples (2 ml) were prepared immediately after collection by centrifuging the samples at 2500 × *g* for 20 min and aspirating the serum. Control serum samples were obtained from 34 adenomatous polyps and 10 healthy volunteers. Fresh tumors and serum were stored at −80°C until further processing.

DNA Isolation. Tumor DNA was isolated and purified with TRIzol reagent (Life Technologies, Inc., NY) according to the manufacturer's instruction. DNA from serum was extracted using a QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) using the blood and body fluid protocol as recommended by the manufacturer (9). Serum (200 μl) was used for DNA extraction with a final elution volume of 50 μl.

MSP. MSP exploits the effect of sodium bisulfate on DNA, which efficiently converts unmethylated cytosine to uracil but which leaves methylated cytosine unchanged. Consequently, after treatment, the methylated and unmethylated alleles have different sequences that can be used to design allele-specific primers (10).

Genomic DNA was modified with the reagents provided in an Intergen CpGenome DNA Modification kit (Intergen Co., Purchase, NY). Serum DNA (20 μl) or tissue DNA (1 μg) was treated with sodium bisulfate following the manufacturer's recommendations.

The modified DNA was then subjected to MSP using CpG WIZ p16 Amplification Kit (Intergen Co.). Primer pairs specific for either the methylated or the modified unmethylated p16 sequences were provided by the kit. With a successfully chemical modification reaction, U Primers amplified only unmethylated DNA, and M Primers amplified only methylated DNA in the region of p16 gene promoter. Each chemically modified experimental DNA sample was amplified with U primers and M

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[3] The abbreviations used are: CRC, colorectal cancer; MSP, methylation-specific PCR.

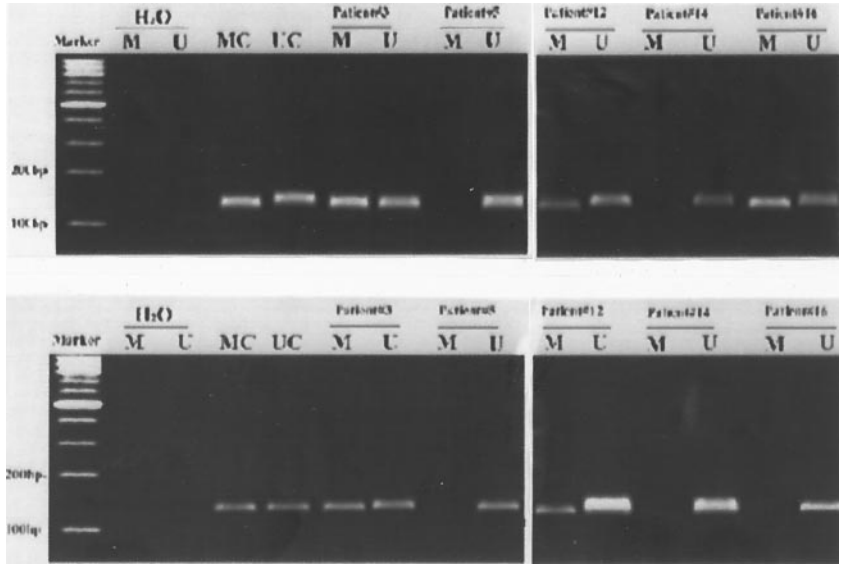

Fig. 1 Detection of aberrant methylation of *p16* gene in the tumors and serum of CRC patients. *Top panel*, tumor samples; *bottom panel*, serum samples; *lane maker*, molecular weight makers; *Lane H_2O*, water blank (negative controls); *Lane M*, methylated p16 sequences (145 bp, *band M*); *Lane U*, unmethylated p16 sequences (154 bp, *band U*); *Lane MC*, positive controls for methylation; and *Lane UC*, positive controls for unmethylation. The detection of a band of 145 bp indicates the presence of methylated p16 sequences in the corresponding tumors or serum of CRC patients.

primers, respectively. The PCR mixture contained 1 × PCR buffer (500 mM KCl, 100 mM Tris-HCl, 1.0% Triton-100, and 15 mM $MgCl_2$), deoxynucleotide triphosphates (each at 0.25 mM), 1 μl of U or M primers, Ampli Taq Gold polymerase (Perkin-Elmer, Foster City, CA), 1U, and 2 μl of bisulfate-modified DNA in a final volume of 30 μl. PCR conditions were as follows: 95°C for 12 min; then 35 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 60 s; and a final extension of 10 min at 72°C. All PCR amplification was performed using a Touchdown thermal cycler (Hybaid, Teddington, England) with tube control for accurate annealing temperatures. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. If the sample contains unmethylated DNA, U primers will produce a 154-bp product (band U). If the sample contains methylated DNA, M primers will produce a 145-bp product (band M). U and M controls provided by the kit served as validation of the reagents and PCR conditions.

Clinicopathological Data and Statistical Analysis. Clinicopathological data were collected after the patients were discharged from the hospital, including age, sex of the patients, anatomical location, Dukes' stage, and differentiation grade. Statistical significance was evaluated by use of the χ^2 test; difference was deemed significant when the $P < 0.05$.

RESULTS

A total of 20 of 52 (38%) tumors were found to have methylated p16 sequences (Fig. 1, *band M*). For the 20 cases with methylated p16 sequences in tumors, MSP was able to detect the same change in the matching serum samples of 14 cases (70%; Fig. 1; Table.1). For the other 32 cases with no detectable p16 methylation in the tumor tissues, no signal was obtained using MSP on matching serum samples. In the population of individuals with p16 methylated tumors, the assay of serum DNA for p16 promoter methylation demonstrated a sensitivity of 70% (14 of 20; 95% confidence interval is 50–90%) and a specificity of 100%. In the total patient population, the sensitivity and specificity are 27 (14 of 52; 95% confidence interval is 15–39%) and 100%, respectively. The association of tumoral p16 methylation status and MSP positivity or negativity in the peripheral serum was statistically significant ($P < 0.0001$).

In the 14 cases with detectable p16 methylation in serum, 10 cases were Dukes' C and D patients. However, in the other 38 patients with no detectable p16 methylation in serum, only 13 cases were Dukes' C and D patients. Dukes' C and D patients were more likely to contain methylated p16 sequences in serum compared with Dukes A and B patients ($P = 0.03$). No correlation was found between p16 methylation and other clinicopathological characteristics.

No methylated p16 sequences were detected in the peripheral serum of 34 patients with adenomatous polyps (Fig. 2) and 10 healthy volunteers. No signals were observed in any of the multiple water blanks.

As a control for the bisulfate modification process, all bisulfate-treated tumor and serum samples were amplified with primers specific for the unmethylated *p16* gene. All samples were found to have amplifiable sequences (band U), thus demonstrating the success of the bisulfate modification process.

DISCUSSION

It is known that double-strand DNA fragments frequently appear in considerable quantities in the serum or plasma of cancer patients. A recent study has shown that concentration of this free DNA varies widely with a mean of 219 ng/ml in the plasma of cancer patients, much higher than ng quantities of DNA circulating in the blood of healthy individuals (11). In our study, even higher DNA amounts (average, 732 ng/ml; data not shown) were found in the serum of CRC patients. Enriched DNA makes it possible to detect tumor-specific DNA alteration in the peripheral blood of patients with cancer.

Serological tumor markers have been proven valuable in the treatment of individuals with cancer for the early detection

Table 1 Detection of p16 methylation in serum of CRC patients with tumor p16 methylation[a]

Case	Age (yrs)	Sex	Serum p16 methylation	Location	Dukes' stage	Grade
3	60	F	+	Distal	C	Moderate
7	73	M	+	Distal	D	Moderate
9	49	M	−	Proximal	C	Poor
12	71	M	+	Distal	C	Moderate
13	56	M	+	Proximal	B	Poor
16	45	M	−	Proximal	B	Moderate
19	35	M	+	Distal	D	Moderate
26	74	F	+	Distal	A	Moderate
27	47	M	+	Proximal	D	Poor
30	70	F	−	Distal	D	Moderate
32	80	F	−	Distal	B	Moderate
33	39	F	+	Distal	C	Moderate
34	51	M	+	Distal	B	Good
37	70	F	+	Proximal	C	Moderate
40	72	M	−	Distal	B	Moderate
44	56	M	+	Distal	D	Moderate
46	47	M	+	Distal	A	Moderate
48	80	F	+	Proximal	C	Poor
49	66	F	+	Distal	D	Moderate
51	39	M	−	Distal	B	Good

[a] Patients (32) with no detectable p16 methylation in tumors were not included in the table.

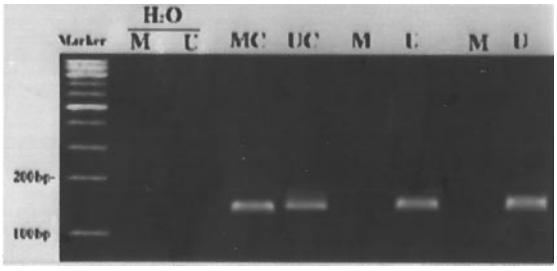

Fig. 2 Detection of aberrant methylation of *p16* gene in the serum of patients with adenomatous polyps. *Lane maker*, molecular weight makers; *Lane H_2O*, water blank (negative controls); *Lane M*, methylated p16 sequences (145 bp, *band M*); *Lane U*, unmethylated p16 sequences (154 bp, *band U*); *Lane MC*, positive controls for methylation; and *Lane UC*, positive controls for unmethylation. None of the serum samples from the patients with adenomatous polyps had detectable methylated p16 sequences.

of primary cancers, early detection of cancer relapse, and as predictors of cancer prognosis (1). Microsatellite instability and loss of heterozygosity have been reported in the serum or plasma from patients with head and neck (12), lung (13), renal (14), and breast (15) cancer but not in the serum of CRC patients (2). Other DNA abnormalities, such as P53 and K-ras mutations, have also been identified in serum or plasma of patients with various cancers (2, 16–18). Moreover, recent studies have demonstrated the presence of gene promoter hypermethylation in the serum or plasma DNA of liver (4, 6), lung (3), head and neck (8), breast (5), and esophageal (7) cancer patients. In this study, we have confirmed the presence of aberrant methylation of the *p16* gene in a significant proportion (38%) of CRC patients and have shown that such an aberration can be detected in the peripheral circulation of CRC patients. This would potentially allow application of this assay for early detection of colon cancers with p16 methylation. The sensitivity of detecting p16 promoter methylation in the serum of CRC patients (27%) is less than the sensitivity reported for the detection of p16 methylation in the serum of liver cancer patients (59%; Ref. 4) and less than the sensitivity reported for detection of K-ras mutation in the serum of CRC patients (43 and 39%; Refs. 16 and 17) and pancreatic cancer patients (81%; Ref. 18) but is higher than the sensitivity reported for detecting p16 methylation in the serum of patients with lung (3), head and neck (8), and breast (5) cancer. Because of relatively low sensitivity of detecting tumor-specific DNA alteration in the peripheral blood of cancer patients, we consider that a multiple altered DNA marker assay can provide a more reliable and informative approach than a single-marker procedure for early detection of cancer patients. Detection of p16 promoter methylation in serum may play a role in cancer detection as a part of a panel of complementary serological markers that included assays for K-ras mutation, p53 mutation, microsatellite instability, and methylated tumor suppressor genes.

In our study, no methylated p16 sequences were found in the serum of patients with no detectable p16 methylation in tumors. Simultaneously, none of the serum samples from the 34 patients with adenomatous polyps and from 10 healthy volunteers had detectable methylated p16 sequences, as is similar to the results reported in the above studies. A specificity of 100% makes the detection of p16 promoter methylation in serum of CRC a more useful serological tumor maker.

Moreover, we also found p16 methylation in serum was significantly associated with later Dukes' stage, which has been considered a most important prognostic determinant. To this point, detection of p16 methylation in the serum should be considered an attractive approach to predict the prognosis of CRC patients. Additionally, our observations suggest that serological detection of aberrant p16 promoter methylation may also be used as a marker for discriminating CRC patients at higher risk for lymph node metastases and distant metastases from

those at lower risk for disseminated disease, but prospective trials should be carried out to further establish these observations.

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