p16 Methylation in Serum as a Potential Marker for the Malignancy of Colorectal Carcinoma

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Abstract. *The aim of the present study was to determine if p16 methylation in the serum can serve as a candidate marker for the malignancy of colorectal carcinoma, real-time quantitative methylation-specific PCR (RTQ-MSP) was performed for p16 methylation in serum and the relationship between p16 methylation levels and clinicopathological findings of colorectal carcinoma was evaluated. Results: The p16 methylation score significantly increased with tumor stage (stage I=0.94±0.47, stage II=2.33±0.90, stage III=8.49±2.37, stage IV=10.03±4.30) (p=0.021; ANOVA). The p16 methylation score was significantly higher in patients with lymph node metastasis (p=0.001) and tumor invasion to the veins (p=0.020). The cumulative survival of patient groups according to the p16 methylation score (more or less than 1) was examined. Interestingly, the group with a high p16 methylation score showed significantly worse survival rates than the group with a low p16 expression score (p=0.006). Conclusion: The p16 methylation score might serve as a new parameter for the prognosis of colorectal carcinoma. Moreover, p16 methylation was found in the serum of patients in all clinical stages, suggesting that early colorectal carcinoma could be detected using the RTQ-MSP method.*

There is now strong evidence that a series of genetic alterations in both dominant oncogenes and tumor suppressor genes are involved in the pathogenesis of human colorectal cancer. Activation of oncogenes such as the *ras* gene, and inactivation of tumor suppressor genes such as the *APC* and *p53* genes, have been identified (1-3). In addition, we found that several other genes are related to the pathogenesis of colorectal cancer (4, 5), and the investigation of genetic changes is important to clarify the tumorigenic pathway (6).

Several tumor suppressor genes contain CpG islands in their promoters, prompting investigation of the role of methylation in silencing these genes. Many tumor suppressor genes show evidence of methylation silencing, providing a new potential pathway for the deactivation of tumor suppressor genes (7). In order to detect primary colorectal cancer early, we examined promoter hypermethylation of the *p16* gene in the serum of patients using methylation-specific PCR (MSP). We found that 13 of 44 (30%) patients with *p16* promoter methylation in their tumor DNA demonstrated abnormal methylation in their serum DNA (8). Subsequently, we examined *p16* methylation using MSP to determine if this technique could be applied to the monitoring of cancer recurrence (9). We detected tumor DNA in the serum of 31 of 45 (69%) patients with recurrent colorectal cancer. These results indicated that promoter methylation in colorectal cancer can be detected in the serum of patients and that this approach can potentially be useful for screening and monitoring of disease.

To determine if *p16* methylation in serum can serve as a candidate marker for the malignancy of colorectal carcinoma, we performed a real-time quantitative methylation-specific PCR (RTQ-MSP) for *p16* methylation in serum and evaluated the relationship between *p16* methylation levels and clinicopathological findings.

Materials and Methods

Sample collection and DNA preparation. Tumor and corresponding normal tissue samples were obtained at the time of surgery from 168 patients with primary colorectal carcinoma at Nagoya University Hospital, Nagoya, Japan. One hundred and sixty-eight corresponding serum samples were obtained one week prior to surgery. Written informed consent, as approved by the

Abbreviations: MSP, methylation-specific PCR; RTQ-MSP, real-time quantitative MSP.

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Key Words: p16, methylation, colorectal carcinoma, prognosis, methylation-specific PCR.

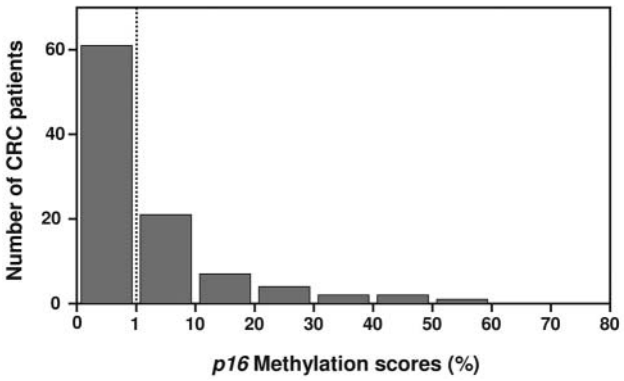

Figure 1. *The distribution of p16 methylation scores in colorectal carcinoma. The median score was 0.37.*

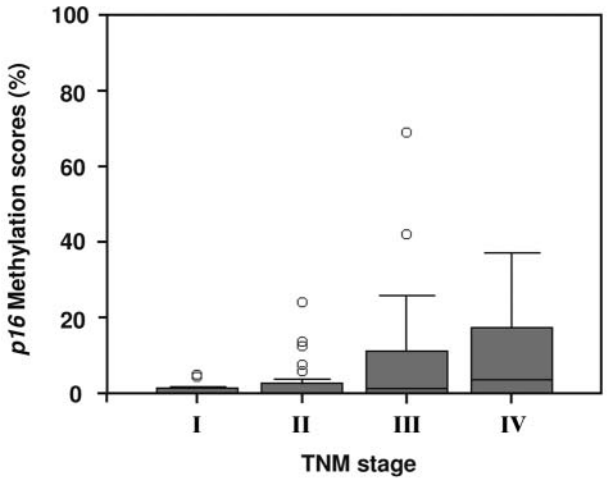

Figure 2. *The distribution of p16 methylation scores according to tumor stage. The upper limits of the boxes and the line across the boxes indicate the 75th percentiles and median, respectively. The upper horizontal bars indicate the maximal scores. Outliers are illustrated as circles. The p16 methylation score significantly increased with the tumor stage (stage I= 0.94±0.47, stage II=2.33±0.90, stage III=8.49±2.37, stage IV=10.03±4.30) (p=0.021; ANOVA).*

institutional review board, was obtained from all patients. Tumor, normal tissue and serum samples were immediately frozen and stored at –80˚C until DNA was extracted. The samples were digested with proteinase K and DNA was prepared as described elsewhere (10).

Bisulfite modification. DNA samples were modified by sodium bisulfite as described elsewhere (11). Briefly, 1 mg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard purification resin (Promega Corp, Madison, WI, USA), treated again with NaOH, precipitated with ethanol, then resuspended in water.

RTQ-MSP. RTQ-MSP was performed in an ABI sequence detection system 7000 using two amplification primers and a dual-labeled fluorogenic hybridization probe (Applied Biosystems, Foster City, CA, USA). The *p16* primers for RTQ-PCR are described elsewhere (12). The sense, antisense primers and fluorogenic probe for the methylated sequence were *p16*MS (5'-TTATTAGAGGGTGGGGCGGATCGC-3'), *p16*MAS (5'-GACCCCGAACCGCGACCGTAA-3'), and *p16*MT [5'-(FAM)-AGTAGTATGGAGTCGGCGGCGGG-(TAMRA)-3'], respectively. The sense, antisense primers and fluorogenic probe for the unmethylated sequence were *p16*US (5'-TTATTAGAG GGTGGGGTGGATTGT-3'), *p16*UAS (5'-CAACCCCAAACCACA ACCATAA-3'), and *p16*UT [5'-(FAM)-AGGTAGTGGGTGG TGGGGAGTAGTATGGAGTTG-(TAMRA)-3'], respectively.

Thermocycling was performed in a final volume of 50 μl containing 5.0 μl of bisulfite-converted DNA from 180 μl of serum sample, 900 nM each of the *p16* primers (sense and antisense), 300 nM of the corresponding fluorogenic probe, 5.0 μl PCR buffer, 2.5 μl dNTP mix, and 1.5 units of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR amplification consisted of 50 cycles (95˚C for 15 s, 60˚C for 60 s and 72˚C for 18 s) after an initial denaturation step (95˚C for 10 min). Multiple negative water blanks were included in each analysis.

Amplification data were analyzed using the Sequence Detection System software (Version 1.6.3) developed by Applied Biosystems. The standard curve was established for quantifying DNA copy number using 9 known copy numbers of serially diluted (1 to 10^8 copies) plasmids containing *p16* methylated or unmethylated DNA. RTQ-MSP assays were conducted in triplicate for each sample and the mean value (Q) was used for further calculation. The concentration (C) of *p16* methylated or unmethylated DNA in serum (genome-equivalents/μl) should be Q/180 because the 5.0 μl of bisulfite-converted DNA applied to RTQ-PCR was derived from 180 μl of serum sample.

p16 Methylation score. The *p16* methylation score in serum was defined as follows: *p16* methylation score (%) = CM / (CM + CU) x 100, where CM = concentration of *p16* methylated DNA in serum and CU = concentration of *p16* unmethylated DNA in serum.

Statistical analysis. The Fisher's exact test was used to examine the possible association between the *p16* methylation score and clinicopathological features. The significance of the correlations between *p16* methylation score and tumor stage (TNM) was determined by one-way ANOVA. Survival rates were calculated by the Kaplan-Meier method for the analysis of censored data. A value of $p<0.05$ (two-tailed) was considered significant. All statistical analyses were performed using the SPSS statistical software package® (SPSS Inc., Chicago, IL, USA).

Results

We first analyzed *p16* methylation levels in 168 colorectal carcinoma and corresponding normal tissue samples using RTQ-MSP. *p16* methylation was detected in 99 of 168 (59%) colorectal carcinoma samples while no corresponding normal tissue samples exhibited *p16* methylation. Subsequently, we examined *p16* methylation levels in the corresponding serum samples of the 99 patients with p16 methylated colorectal carcinomas. Figure 1 shows the distribution of *p16*

Table I. *Clinicopathological features and p16 methylation scores in colorectal carcinoma patients.*

Clinicopathological features	Variable	No. of patients	*p16* methylation score (mean±S.D.)	*p*-value[a]
Age	≤65	51	5.6±9.6	0.772
	65<	48	5.7±12.7	
Gender	male	58	4.1±8.0	0.571
	female	41	8.0±14.8	
Max. tumor size (cm)	≤5.0	51	3.1±8.6	0.052
	5.0<	48	7.8±12.7	
Histology	tub[b]	89	5.0±9.1	0.550
	por[c], muc[d]	10	9.7±22.5	
Depth of tumor	≤mt[e]	21	3.0±9.8	0.190
	mt<	78	6.0±11.3	
Lymph node metastasis	+	48	9.2±14.3	**0.001**
	–	51	1.9±4.4	
Tumor invasion to veins	+	40	9.3±15.1	**0.020**
	–	59	2.9±6.0	

[a]Fisher's exact test; [b]tub, tubular adenocarcinoma; [c]por, poorly-differentiated adenocarcinoma; [d]muc, mucinous adenocarcinoma; [e]mt, muscular tunic.

methylation scores of these patients. The median score was 0.37. All serum samples also exhibited unmethylated *p16*, confirming the quality of DNA extracted from serum. As a control, serum samples from 30 healthy volunteers were also subjected to bisulfite conversion and RTQ-MSP. No *p16* methylation was detected in these samples.

Figure 2 shows the *p16* methylation scores of 99 patients with *p16* methylated colorectal carcinomas according to TNM stage. The *p16* methylation score significantly increased with the tumor stage (stage I=0.94±0.47, stage II=2.33±0.90, stage III=8.49±2.37, stage IV=10.03±4.30) (p=0.021; ANOVA). We next correlated the *p16* methylation score and clinicopathological features. The *p16* methylation score was significantly higher in patients with lymph node metastasis (p=0.001) and tumor invasion to the veins (p=0.020). These results are summarized in Table I. Taken together, the *p16* methylation level in the serum was closely associated with the malignancy of colorectal carcinoma. In addition, early colorectal carcinoma (stage I) could be detected by RTQ-MSP, suggesting that this technique can be applied for the early detection of this carcinoma.

We then examined the cumulative survival of patients according to the *p16* methylation score (methylation score; more or less than 1). Interestingly, the group with a high *p16* methylation score had significantly worse survival rates than the group with a low *p16* expression score (Figure 3, p=0.006). This result also supports the idea that *p16* methylation in serum could be a novel marker for the malignancy of colorectal carcinoma.

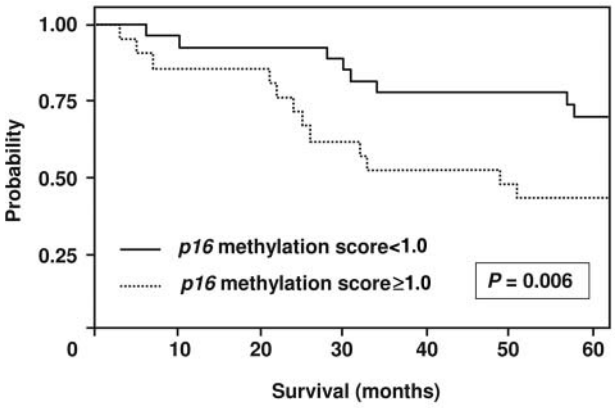

Figure 3. *The cumulative survival of patient groups according to p16 methylation scores (methylation score; more or less than 1). The high p16 methylation score group showed significantly worse survival rates than the low p16 methylation score group (p=0.006).*

Discussion

Colorectal carcinoma is one of the most aggressive types of cancer and occurs at a high incidence in most countries (13). Thus, it is important to determine if genetic alterations can aid in the estimation of colorectal carcinoma malignancy.

Several studies have identified tumor-specific genetic alterations in the plasma and serum DNA of cancer patients (14-16). Microsatellite shift and loss of heterozygosity, for example, have been detected in the plasma and serum DNA of patients with head and neck carcinoma, and small cell lung carcinoma (17, 18). Mutant K-*ras* and *p53* have also been identified in the plasma of patients with pancreatic cancer (19). In colorectal cancers, Hibi *et al.* suggested that K-*ras* or *p53* mutations could be detected in the circulating tumor DNA from the serum of colorectal cancer patients using the mismatch ligation assay (20). However, screenings based on the results of this study are limited by the large number of different mutations identified in the *p53* gene. Therefore, we searched for a technique that would allow for the detection of tumor DNA in the serum without requiring advance knowledge of the position of genetic alterations.

The RTQ-MSP technique has several advantages in detecting tumor DNA in the serum of cancer patients. First, its high sensitivity makes it possible to detect even small amounts of tumor DNA in the serum. This technique also has great specificity, and no abnormal methylation in the serum is observed if the corresponding tumors do not exhibit methylation. Although *p16* methylation of serum DNA is not specific for colorectal cancer and does not indicate the origin of the tumor, RTQ-MSP can be applied to detect a wide variety of tumors that have shown aberrant methylation in the gene promoter. Thus, knowledge of the

methylation status of the primary tumor is not required in advance to detect circulating tumor DNA, suggesting that RTQ-MSP can be used as a screening method for cancer.

In this study, we demonstrated for the first time that *p16* methylation in the serum increased with the tumor stage of colorectal carcinoma and was associated with a poor prognosis. These results suggest that the *p16* methylation score might serve as a new parameter for the prognosis of colorectal carcinoma. Moreover, *p16* methylation was found in the serum of patients of all clinical stages, suggesting that early colorectal cancer could be detected using the RTQ-MSP method.

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