Detection of Vimentin (*VIM*) Methylation in the Serum of Colorectal Cancer Patients

ATSUSHI SHIRAHATA, KAZUMA SAKURABA, TETSUHIRO GOTO, MITSUO SAITO, KAZUYOSHI ISHIBASHI, GAKU KIGAWA, HIROSHI NEMOTO and KENJI HIBI

Gastroenterological Surgery, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama 227-8501, Japan

Abstract. *Aim: Detection of colorectal cancer using serum assay of vimentin methylation. Materials and Methods: We attempted to detect vimentin methylation in the serum of colorectal cancer patients using quantitative methylation-specific polymerase chain reaction (qMSP). Results: Of 44 colorectal cancer patients, 4 (9%) exhibited methylation of the vimentin gene in their serum DNA by qMSP. Interestingly, methylation was significantly found in the serum of patients with liver metastasis, peritoneal dissemination, and distant metastasis (p=0.026, p=0.0029 and p=0.0063, respectively), suggesting that vimentin methylation in serum might be detected more frequently in patients with advanced colorectal cancer. Conclusion: The high sensitivity of qMSP makes it possible to detect smaller amounts of tumor DNA in the serum, suggesting that qMSP can be used as a screening method for cancer.*

Previous studies have proposed that enriched circulating DNA can be found in the serum of cancer patients (1, 2). On the basis of these studies, many attempts have been made for the early detection of tumor-related aberrant DNA in the serum of patients with various malignancies (3, 4). Our studies have also shown that it is possible to detect tumor-specific DNA in the serum of various cancer patients using a mismatch ligation assay for K-*ras* and mitochondrial DNA mutations (5-8).

Recent studies have indicated that promoter methylation is an important mechanism for gene transcriptional inactivation. Others and we have previously found that several genes such as *p16* (9), *p14* (10), helicase-like transcription factor (*HLTF*) (11), suppressor of cytokine signaling-1 (*SOCS-1*) (12), and cadherin 13 (*CDH13*) (13) harbored promoter hypermethylation associated with a loss of gene expression in digestive tract cancer. The presence of epigenetic methylation might be useful as a molecular target for the detection of tumor DNA.

Previously, we examined the methylation status of *p16* in colorectal cancer using methylation-specific PCR (MSP) (14). DNA of 44 out of 94 (47%) tumors exhibited abnormal promoter methylation of the *p16* gene. Subsequently, we examined whether aberrant methylation could be detected in corresponding serum DNA, and found that 13 out of 44 (30%) patients with *p16* promoter methylation in tumor DNA demonstrated abnormal methylation in their serum DNA. This result encouraged us in an attempt to detect methylation in serum DNA using a molecular biological technique.

Recently, it was shown that the vimentin gene (*VIM*), usually activated in mesenchymal cells, was highly methylated in colorectal carcinoma (15). Several reports indicated that *VIM* gene methylation was detected 53-84% of colorectal carcinomas (16-18). Moreover, *VIM* methylation can be applied for screening or as a diagnostic tool of colorectal carcinoma in a fecal DNA test. In this study, we attempted to detect *VIM* methylation in the serum of colorectal cancer patients.

Materials and Methods

Sample collection and DNA preparation. Tumor samples were obtained at the time of surgery from 44 patients with primary colorectal carcinoma at Showa University Fujigaoka Hospital, Yokohama, Japan. Forty-four corresponding serum samples had been obtained 1 week prior to surgery. Written informed consent, as indicated by the Institutional Review Board, was obtained from all patients. Tumor 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 previously (19).

Sodium bisulfite modification. One μg of the genomic DNA extracted from the tumor specimens and 200 μl of the corresponding serum were subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany).

Correspondence to: Kenji Hibi, Gastroenterological Surgery, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama 227-8501, Japan. Tel: +81 459711151, Fax: +81 459717125, e-mail: kenjih-ngy@umin.ac.jp

Key Words: VIM, quantitative methylation-specific PCR, colorectal carcinoma.

Table I. *Clinicopathological features of colorectal cancer partients with VIM methylation in serum.*

Case no.	Age (years)	Gender	Histology	TNM classification				Peritoneal dissemination	Liver metastasis	Distant metastasis	Duke's stage
				T	N	M	Stage				
8	55	Female	Well[a]	T3	N0	M1	IV	–	+	+	C
25	65	Male	Well	T3	N1	M1	IV	+	+	–	C
27	49	Male	Poor[b]	T4	NX	M1	IV	–	+	+	C
42	62	Female	Well	T3	N1	M0	IIIB	–	–	–	C

[a]Well: Well-differentiated carcinoma; [b]Poor: poorly differentiated adenocarcinoma.

Quantitative methylation-specific polymerase chain reaction (qMSP). The bisulfite-treated DNA was amplified with qMSP, using a Thermal Cycler Dice® Real- Time System TP800 (Takara Bio Inc., Otsu, Japan). Thermocycling was performed in a final volume of 25 μl containing 2.0 μl of the serum DNA sample, 100 nM of each of the *VIM* or β-actin (*ACTB*) primers (forward and reverse), and 12.5 μl of SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Japan), which consisted of Taq DNA polymerase, reaction buffer and deoxynucleotide triphosphate mixture. The qMSP primer sequences for *VIM* have been described elsewhere (15) and were: *VIM* MS (sense), 5'-TCGTTTCGAGGTTTTCGCGTTAGAGAC-3', and *VIM* MAS (antisense), 5'-CGACTAAAACTCGACCG ACTCGCGA-3'. The PCR amplification consisted of 40 cycles (95˚C for 5 s and 60˚C for 30 s) after an initial denaturation step (95˚C for 10 s). The bisulfite-treated DNA obtained from L132 cells that was fully methylated by *SssI* methylase was used as a positive control. To correct for differences in both quality and quantity between samples, *ACTB* was used as an internal control. The targets were obtained from the same bisulfite-treated DNA.

Statistical analysis. We evaluated the correlation between the *VIM* methylation status of serum samples and their clinicopathological characteristics. Statistical significance was evaluated by Chi-square test or Student's *t*-test. A *p*-value <0.05 indicated statistical significance.

Results

We first examined the methylation status of *VIM* in the tumor DNA of colorectal cancer patients. Of 44 colorectal cancer patients, 27 (61%) exhibited a methylation of tumor *VIM* by qMSP.

We next examined the methylation status of *VIM* in the serum DNA of colorectal cancer patients. Of 44 colorectal cancer patients, 31 (70%) exhibited a methylation of *VIM* in their serum DNA by qMSP (Figure 1). However, 10 out of 31 patients exhibited no *VIM* methylation in their tumors, suggesting that the identification of *VIM* methylation in the serum of these 10 patients was falsely positive. Therefore, we considered that the findings of *VIM* methylation in serum derived from four colorectal cancer patients who also exhibited *VIM* methylation in their tumor and a higher methylation level in their serum than these 10 patients were truly positive (Figure 1) (Table I).

Table II. *Clinicopathological features and VIM methylation in serum DNA of colorectal cancer partients.*

		VIM methylation		
	No.	+	–	*p*-Value
Age (years, mean±S.D.)	44	57.8±7.2	66.2±12.4	0.19**
Gender				
Male	23	2	21	0.92*
Female	21	2	19	
Depth of tumor				
Mt≥	12	0	12	0.19*
Mt<	32	4	28	
Lymph node metastasis				
+	20	3	17	0.21*
–	24	1	23	
Liver metastasis				
+	6	2	4	0.026*
–	38	2	36	
Peritoneal dissemination				
+	5	3	2	0.0029*
–	39	1	38	
Distant metastasis				
+	2	2	0	0.0063*
–	42	2	40	
Duke's stage				
A	8	0	8	0.111*
B	14	0	14	
C	22	4	18	
Total	44	4	40	

*Chi-square test; **Student's *t*-test; Mt, muscular tunic.

After completion of qMSP analysis in all specimens, clinicopathological data were correlated with the molecular analysis (Table II). We found no association of overall methylation in the serum DNA with age, gender, extent of tumor, or presence of lymph node metastasis. Interestingly, methylation was significantly found in the serum of patients with liver metastasis, peritoneal dissemination, and distant metastasis (p=0.026, p=0.0029 and p=0.0063, respectively), suggesting that *VIM* methylation in serum might be detected more frequently in patients with advanced colorectal cancer.

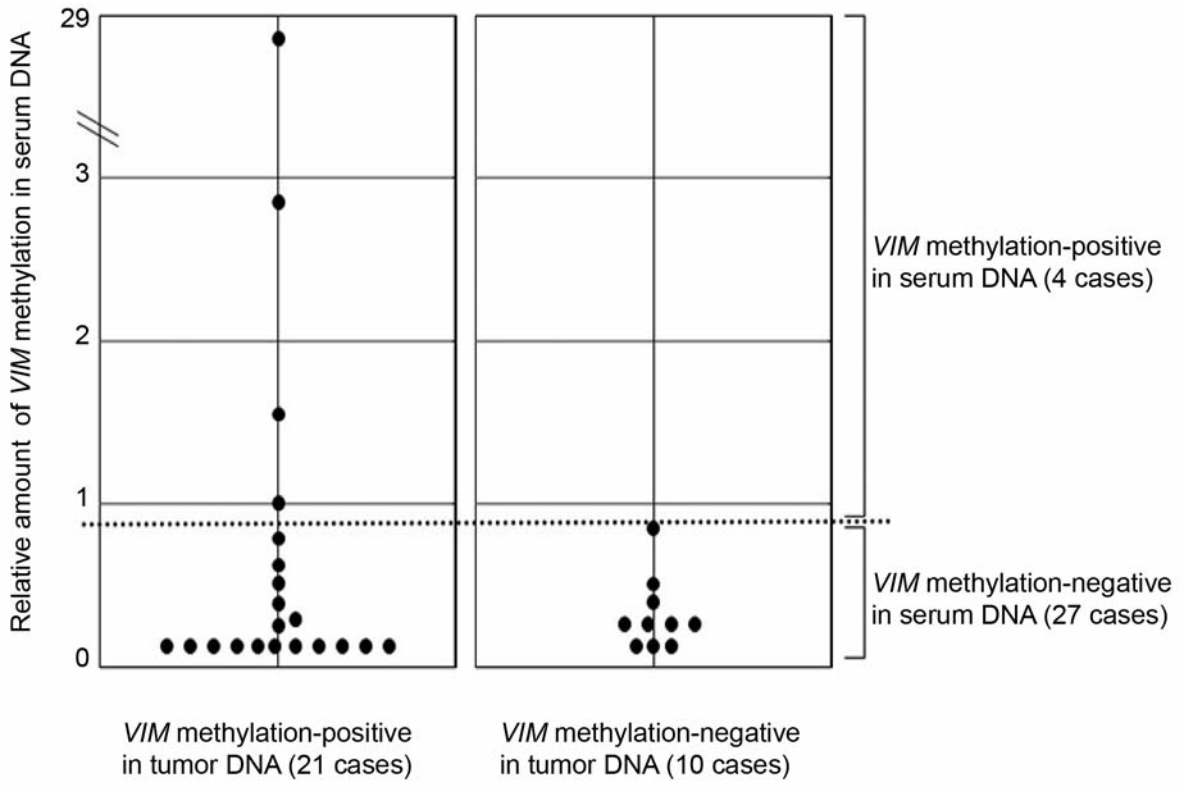

Figure 1. *Of 44 colorectal cancer patients, 31 (70%) exhibited a methylation of VIM in their serum DNA by qMSP. However, 10 out of these 31 patients exhibited no VIM methylation in their tumors. Therefore, we considered that the findings of VIM methylation in serum derived from 4 colorectal cancer patients who also exhibited VIM methylation in their tumor and a higher methylation level in their serum than these 10 patients were truly positive.*

Discussion

In previous studies, tumor-related aberrant DNA has been identified in the serum of cancer patients. In particular, methylation of the *p16* tumor suppressor gene has been studied in various malignancies such as esophageal cancer, colorectal cancer, non-small cell lung cancer, liver cancer, and head and neck cancer (14, 20-24). For the detection of promoter methylation in these types of cancer, the MSP technique is a beneficial procedure because of its high sensitivity and specificity. MSP has sufficient sensitivity to detect even a very low level of tumor DNA in serum, having detected nearly 1 methylated gene copy per 1000 unmethylated copies in dilution experiments (25).

qMSP has several advantages in detecting tumor DNA in the serum of cancer patients. Its high sensitivity makes it possible to detect smaller amounts of tumor DNA in the serum. This technique also has great specificity, and no abnormal methylation in serum has yet been observed if corresponding tumors do not exhibit methylation (14). Thus, in the present study, we suggest that the identification of *VIM* methylation in the serum of 10 out of 31 patients who exhibited no *VIM* methylation in their tumor was falsely positive. In addition, we considered that the identification of *VIM* methylation in the serum derived from 4 colorectal cancer patients who exhibited *VIM* methylation in their tumor and higher methylation level in their serum than these 10 patients were truly positive for methylation. This observation supports the idea that qMSP could be applicable to clinical use in the detection of tumor DNA in serum. Although *VIM* methylation in serum DNA is not specific for colorectal cancer and thus does not indicate that the colorectum is the location of the primary tumor, qMSP can be used to detect a wide variety of tumors that have shown aberrant methylation in the promoters of genes. In principle, the methylation status of a primary tumor is not required in advance to detect circulating tumor DNA, suggesting that qMSP can be used as a screening method for cancer.

In conclusion, our results suggest that *VIM* methylation might be a good marker for the detection of tumor DNA in the serum of colorectal carcinoma patients. Although the population used in this study was small, and further examination will be necessary in future, these results suggest that *VIM* methylation in serum may serve as a new marker for the monitoring and screening of colorectal carcinoma.

Acknowledgements

We would like to thank M. Ogata for her technical assistance.

References

1 Leon SA, Shapiro B, Sklaroff DM and Yaros MJ: Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res *37*: 646-650, 1977.
2 Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C and Beljanski M: Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology *46*: 318-322, 1989.
3 Hibi K, Robinson CR, Booker S, Wu L, Hamilton SR, Sidransky D and Jen J: Molecular detection of genetic alterations in the serum of colorectal cancer patients. Cancer Res *58*: 1405-1407, 1998.
4 Mulcahy HE, Lyautey J, Lederrey C, Qi Chen X, Anker P, Alstead EM, Ballinger A, Farthing MJ and Stroun M: A prospective study of K-*ras* mutations in the plasma of pancreatic cancer patients. Clin Cancer Res *4*: 271-275, 1998.
5 Ito S, Hibi K, Nakayama H, Kodera Y, Ito K, Akiyama S and Nakao A: Detection of tumor DNA in serum of colorectal cancer patients. Jpn J Cancer Res *93*: 1266-1269, 2002.
6 Uemura T, Hibi K, Kaneko T, Takeda S, Inoue S, Okochi O, Nagasaka T and Nakao A: Detection of oncogenic mutation in the plasma DNA of pancreatic cancer patients. J Gastroenterol *39*: 56-60, 2004.
7 Hibi K, Nakayama H, Yamazaki T, Takase T, Taguchi M, Kasai Y, Ito K, Akiyama S and Nakao S: Detection of mitochondrial DNA alterations in primary tumors and corresponding serum of colorectal cancer patients. Int J Cancer *94*: 429-431, 2001.
8 Okochi O, Hibi K, Uemura T, Inoue S, Takeda S, Kaneko T and Nakao A: Detection of mitochondrial DNA alterations in the serum of hepatocellular carcinoma patients. Clin Cancer Res *8*: 2875-2878, 2002.
9 Myohanen SK, Baylin SB and Herman JG: Hypermethylation can selectively silence individual $p16^{ink4A}$ alleles in neoplasia. Cancer Res *58*: 591-593, 1998.
10 Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB and Herman JG: Hypermethylation-associated inactivation of *p14* (ARF) is independent of *p16* (INK4a) methylation and *p53* mutational status. Cancer Res *60*: 129-133, 2000.
11 Hibi K, Nakayama H, Kanyama Y, Kodera Y, Ito K, Akiyama S and Nakao A: Methylation pattern of *HLTF* gene in digestive tract cancers. Int J Cancer *104*: 433-436, 2003.
12 Fujitake S, Hibi K, Okochi O, Kodera Y, Ito K, Akiyama S and Nakao A: Aberrant methylation of *SOCS-1* was observed in younger colorectal cancer patients. J Gastroenterol *39*: 120-124, 2004.
13 Hibi K, Nakayama H, Kodera Y, Ito K, Akiyama S and Nakao A: CDH13 promoter region is specifically methylated in poorly differentiated colorectal cancer. Br J Cancer *90*: 1030-1033, 2004.
14 Nakayama H, Hibi K, Taguchi M, Takase T, Yamazaki T, Kasai Y, Ito K, Akiyama S and Nakao A: Molecular detection of *p16* promoter methylation in the serum of colorectal cancer patients. Cancer lett *188*: 115-119, 2002.
15 Itzkowitz SH, Jandorf L, Brand R, Rabeneck L, Schroy PCIII, Sontag S, Johnson D, Skoletsky J, Durkee K, Markowitz S and Shuber A: Improved fecal DNA test for colorectal cancer screening. Clin Gastroenterol Hepatol *5*: 111-117, 2007.
16 Zou H, Harrington JJ, Shire AM, Rego RL, Wang L, Campbell ME, Oberg AL and Ahlquist DA: Highly methylated genes in colorectal neoplasia: implications for screening. Cancer Epidemiol Biomarkers Prev *16*: 2685-2696, 2007.
17 Brenner DE and Rennert G: Fecal DNA biomarkers for the detection of colorectal neoplasia: Attractive, but is it feasible? J Natl Cancer Inst *97*: 1107-1109, 2005.
18 Zou H, Harrington J, Rego RL and Ahlquist DA: A novel method to capture methylated human DNA from stool: Implications for colorectal cancer screening. Clin Chem *53*: 1646-1651, 2007.
19 Hibi K, Nakamura H, Hirai A, Fujikake Y, Kasai Y, Akiyama S, Ito K and Takagi H: Loss of H19 imprinting in esophageal cancer. Cancer Res *56*: 480-482, 1996.
20 Hibi K, Taguchi M, Nakayama H, Takase T, Kasai Y, Ito K, Akiyama S and Nakao A: Molecular detection of *p16* promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin Cancer Res *7*: 3135-3138, 2001.
21 Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB and Herman JG: Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res *59*: 67-70, 1999.
22 Wong IH, Lo YM, Zhang J, Liew CT, Ng MH, Wong N, Lai PB, Lau WY, Hjelm NM and Johnson PJ: Detection of aberrant *p16* methylation in the plasma and serum of liver cancer patients. Cancer Res *59*: 71-73, 1999.
23 Nakayama H, Hibi K, Takase T, Yamazaki T, Kasai Y, Ito K, Akiyama S and Nakao A: Molecular detection of *p16* promoter methylation in the serum of recurrent colorectal cancer patients. Int J Cancer *105*: 491-493, 2003.
24 Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG and Sidransky D: Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res *60*: 892-895, 2000.
25 Vogelstein B and Kinzler KW: Digital PCR. Proc Natl Acad Sci USA *96*: 9236-9241, 1999.

Received September 4, 2010
Revised October 18, 2010
Accepted October 19, 2010