Serum Vimentin Methylation as a Potential Marker for Colorectal Cancer

ATSUSHI SHIRAHATA[1,2] and KENJI HIBI[1]

[1]*Gastroenterological Surgery, Showa University Fujigaoka Hospital, Aoba-ku, Yokohama, Japan;*
[2]*Gastroenterological Surgery, Yokohama Asahi Chuo General Hospital, Aoba-ku, Yokohama Japan*

Abstract. *Aims: The present study aimed to examine the feasibility of detecting vimentin (VIM) methylation in the serum of patients with colorectal cancer (CRC) and to determine the effectiveness of a relatively simple, inexpensive, and non-invasive test performed in combination with the conventional carcinoembryonic antigen analysis. Materials and Methods: VIM methylation in the serum DNA of 242 patients with CRC was measured by a quantitative methylation-specific polymerase chain reaction. Results: A significantly higher positive rate was obtained for VIM methylation than for carcinoembryonic antigen or carbohydrate antigen 19-9 in stage 0, I, and II patients. The combination of all three markers yielded similar sensitivity for patients with disease of stage 0: 57.1%, I: 36.1%, II: 45.2%, and III: 55.4%, whereas the sensitivity reached 85.7% for patients with stage IV disease. Conclusion: VIM methylation of serum DNA may be a useful marker for the early detection of CRC.*

More than 945,000 new cases of colorectal cancer (CRC) are reported every year, and approximately 492,000 patients die of the disease annually (1, 2). Despite advances in CRC treatment, therapeutic efficacy has plateaued, making early diagnosis fundamental for the control of morbidity and mortality, especially since patients diagnosed with early stage disease often show improved long-term survival (3).

Recently, vimentin (*VIM*), which is usually activated in mesenchymal cells, has been shown to be highly methylated in CRC (4-8). Studies indicate that the detection of *VIM* methylation could be useful as either a screening or a diagnostic tool for CRC in various times of clinical samples (7, 8), and a molecular biological technique to detect methylated DNA in serum should therefore be developed. Herein, we aimed to examine the feasibility of detecting *VIM* methylation in the serum of patients with CRC and determine the effectiveness of a relatively simple, inexpensive, and noninvasive test performed in combination with the more conventional carcinoembryonic antigen (CEA) analysis for the early detection of CRC (9-11).

Correspondence to: Atsushi Shirahata, Department of Gastroenterological Surgery, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama 227-8501, Japan. Tel: +81 459711151, Fax: +81 459717125, e-mail: shirahataa@asahi-hp.jp

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Materials and Methods

Sample collection and DNA preparation. The present case–control study included 25 healthy donors and 242 patients with CRC. Written informed consent, as per the guidelines of the Institutional Review Board of Showa University Fujigaoka Hospital, Yokohama, Japan, was obtained from all patients.

Blood samples from 242 enrolled patients with CRC were obtained one week before surgery at the Showa University Fujigaoka Hospital, Yokohama, Japan, whereas those from the 25 healthy donors were obtained at the hematology unit of the same hospital. Serum was separated by centrifugation of peripheral blood at 700 × *g* for 15 min, then immediately frozen and stored at −80˚C until DNA extraction. Serum samples were digested with proteinase K, and DNA was prepared as described previously (12).

The clinicopathological profiles of all patients enrolled in this study are shown in Table I. Normal levels of CEA and carbohydrate antigen 19-9 (CA19-9) were set at <5 ng/ml and <37 U/ml, respectively. Depth of tumor invasion (T), lymph node metastasis (N), distant metastasis (M), and tumor stage were determined according to the current tumor-node-metastasis (TNM) classification of malignant tumors by the International Union against Cancer (2009 revision) (13).

Sodium bisulfite modification. DNA extracted from 200 μl of serum was subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany), eluted with 20 μl of elution buffer, and stored at −80˚C.

Quantitative methylation-specific polymerase chain reaction. Bisulfite-treated DNA samples were amplified by quantitative methylation-specific polymerase chain reaction (qMSP) in 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 each of the *VIM* or β-actin (*ACTB*) primers (forward and reverse), and 12.5 μl

Table I. *Clinicopathological features and serum VIM methylation in patients with colorectal cancer.*

		VIM methylation in serum DNA		
	No	Negative	Positive	*p*-Value
Age (years), mean serum DNA	242	67.48	67.04	0.778††
Gender				
Male	140	92	58	0.130†
Female	102	72	30	
CEA (ng/ml)				
5>	161	110	51	0.795†
5≤	81	54	27	
CA19-9 (U/ml)				
37>	204	138	66	0.925†
37≤	38	26	12	
Histology				
Well-differentiated	196	134	62	0.438†
Poorly differentiated	46	30	18	
Location				
Proxymal colon	82	53	29	0.673†
Distal colon	64	43	21	
Rectum	96	68	28	
Size				
45>	123	82	41	0.693†
45≤	110	76	34	
Unknown	9	6	3	
Depth of tumor invasion				
Tis, T1	25	15	10	0.0993†
T2	32	24	8	
T3	123	89	34	
T4a	51	30	21	
T4b	8	3	5	
TX	3	3	0	
Lymph node metastasis				
N0, N1a, N1b	207	145	62	0.0479†
N2a, N2b	29	15	14	
NX	6	4	2	
Distant metastasis				
M0	191	129	62	0.980†
M1a, M1b	49	33	16	
MX	2	2	0	
TNM Stage				
0	7	4	3	0.773†
I	36	25	11	
IIA, IIB, IIC	73	52	21	
IIIA, IIIB, IIIC	74	48	26	
IVA, IVB	49	33	16	
Unknown	3	3	0	
Dukes' stage				
A	44	29	15	0.944†
B	71	50	21	
C	77	51	26	
D	50	34	16	
Total	242	164	78	

††Chi-square test; Studentare †*t*-test; VIM: vimentin; TNM: tumor-node-metastasis; CEA: carcinoembryonic antigen.

of SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Japan), which included Taq DNA polymerase, reaction buffer, and deoxynucleotide triphosphate mixture. The qMSP primer sequences for *VIM* have been described in another study (5) and were as follows: *VIM* MS (sense): 5'-TCGTTTCGAGGTTTTCGCGTTAGAGAC-3', and *VIM* MAS (antisense): 5'-CGACTAAAACTCGACCGACTCGCGA-3'. The polymerase chain reaction amplification protocol comprised 40 cycles (95˚C for 5 s and 55˚C for 30 s), after an initial denaturation step (95˚C for 10 s).

Bisulfite-treated DNA obtained from L132 cells was completely methylated by SssI methylase and used as the positive control. To adjust for quality and quantity differences among samples, β-actin (*ACTB*) was used as an internal control. The targets were obtained from the same bisulfite-treated DNA. *VIM* methylation was defined as positive if the relative amount of *VIM*-methylated DNA in serum (normalized to the internal control) was >0.0485.

Statistical analysis. The relative amounts of *VIM* methylation in serum DNA were reported as mean±standard deviation, and their distributions in patients with CRC and healthy donors were compared using the Mann–Whitney *U*-test. *VIM* methylation was considered positive if the methylation value of a sample was higher than the cutoff point. The correlation between patients' *VIM* methylation status and their clinicopathological characteristics was evaluated using the chi-square test or Student's *t*-test. Serum marker sensitivity at various tumor stages was compared using the chi-square test. A *p*-value of <0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences version 13.0 for Windows, release 15, 2006 (SPSS Inc., Chicago, IL, USA).

Results

VIM methylation in serum DNA in patients with CRC and healthy donors. The mean level of *VIM* methylation in the DNA samples of patients with CRC was 0.137±0.38 (range=0-4.10), whereas that of the DNA from healthy donors was 0.011±0.015 (range=0-0.048). No statistically significant difference was observed between the two groups (p=0.181) (Figure 1).

Clinicopathological features and VIM methylation status in serum DNA of patients with CRC. After completing the qMSP analysis in all samples, clinicopathological data were correlated with the molecular analysis results (Table I). There was no association between serum *VIM* methylation levels and age, sex, CEA level, CA19-9 level, histology, location, size, depth of tumor invasion, distant metastasis, TNM stage, or Dukes' stage. However, patients with *VIM* methylation significantly more frequently had lymph node metastasis (p=0.0479). Moreover, a trend towards a correlation between increasing invasion depth of the primary tumor and *VIM* methylation (p=0.093) was observed, suggesting that *VIM* methylation of serum DNA may be indicative of the malignant potential of CRC.

Interestingly, *VIM* methylation was detected in the serum of patients with CRC at every disease stage. The sensitivity of the test was similar for patients with stage 0 (57.1%), I

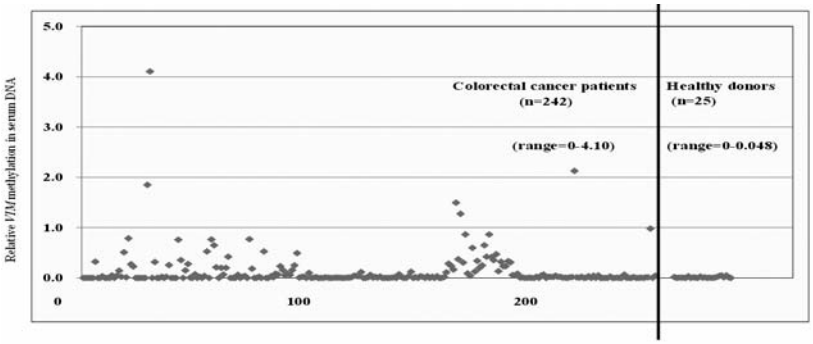

Figure 1. *Distribution of the relative vimentin methylation levels of serum DNA from patients with colorectal cancer and healthy donors (mean±SD) (0.137±0.38 vs. 0.011±0.015, p=0.181).*

Table II. *The sensitivity of serum markers for colorectal cancer according to the tumor stage.*

	Stage 0 (n=7)	Stage I (n=36)	Stage II (n=73)	Stage III (n=74)	Stage IV (n=49)	Total (n=239)
CEA	1 (14.3%)	2 (5.6%)	13 (17.8%)	27 (36.5%)	36 (73.5%)	79 (33.1%)
CA19-9	0 (0%)	0 (0%)	8 (11.0%)	9 (12.2%)	20 (40.8%)	37 (15.5%)
VIM methylation	4 (57.1%)	11 (30.6%)	21 (28.8%)	26 (35.1%)	16 (32.7%)	78 (32.6%)
CEA+CA19-9+*VIM* methylation	4 (57.1%)	13 (36.1%)	33 (45.2%)	41 (55.4%)	42 (85.7%)	133 (55.6%)

VIM: Vimentin; CEA: carcinoembryonic antigen.

(30.6%), II (28.8%), III (35.1%), and IV (32.7%) diseases, suggesting that the qMSP technique could be used for detecting CRC at an early disease stage.

A combination of serum markers for the early detection of CRC. The sensitivity of the test for detecting *VIM* methylation in serum DNA of patients with CRC was compared to that of CEA and CA19-9 detection tests. Cases with staging difficulties (n=3) were excluded. The cut-off values for *VIM* methylation, CEA, and CA19-9 were determined to be 0.0485, 5 ng/ml, and 37 U/ml, respectively.

Table II shows the sensitivity of each marker. The sensitivity of *VIM* methylation as a marker was 32.6%, which was comparable to that of CEA (33.1%) and higher than that of CA19-9 (15.5%). The sensitivity of *VIM* methylation was similar for patients at all disease stages, whereas that of CEA and CA19-9 gradually increased as tumor stage advanced. Although the sensitivity of *VIM* methylation was lower than those of CEA and CA19-9 for patients with stage IV disease, it was comparable to that of CEA for stage III, and higher than those of CEA and CA19-9 for the other stages. Furthermore, the positivity rate of *VIM* methylation was significantly higher than those of CEA and CA19-9 for stage 0, I, and II patients (stage 0: p=0.033, stage I: p=0.00012, stage II: p=0.023).

Next, we examined the combination of all three serum markers as a method for early CRC detection with improved sensitivity. For patients at every stage of disease, the sensitivity of the combination was higher than that of any single marker (Table II). In addition, the combination's sensitivity was similar in patients with stage 0 to III disease (~36-57%), while reaching 85.7% in patients with stage IV disease (Table II).

Discussion

The risks of recurrence of CRC and subsequent death are closely related to the disease stage at the time of primary diagnosis. In other words, early detection of cancer and high-risk precursor lesions will improve survival rates (3). The detection of CRC at an earlier early stage requires the development of novel, less-invasive screening methods. Serum biomarkers have the potential to significantly enhance mass screening programs because they can be detected and analyzed in a relatively noninvasive and economical manner (14-18). Tumor-associated antigens, such as CEA and CA19-9, which are commonly used in the management of CRC, have been investigated as serum markers. However, they often demonstrate low sensitivity, suggesting that they cannot be used efficiently for screening and diagnosis, and that their

use should be limited to post-surgical surveillance (9). Therefore, it is important to identify novel candidate serum biomarkers for the early detection of CRC.

VIM, a member of the intermediate filament protein family, exhibits a complex gene expression pattern that can be observed at several levels (19). VIM is first expressed during embryonic development in the mesodermal cells located between the primitive streak and the proximal endoderm. Many tissue types originating from this region continue to express VIM after differentiation. Moreover, VIM has been suggested to act as a signal transducer, relaying information from the extracellular matrix to the nucleus (20). In 2005, Chen *et al*. first reported that the exon 1 region of *VIM* is relatively hypermethylated in CRC compared to the normal colorectal mucosa and other normal tissues (4). Several reports that followed indicated that *VIM* methylation was detected in 53-84% of CRCs (5-8). Additionally, in a previous study, we detected aberrant methylation of *VIM* in 31 out of 48 (65%) primary CRCs (6). Thus, the frequent methylation of *VIM* in CRC raises the possibility that the detection of such a feature in clinical samples, including serum or stool samples, could be employed to screen for this malignancy. These findings prompted us to examine the methylation status of *VIM* in the serum DNA obtained from patients with CRC.

The qMSP technique is a valuable method for detecting promoter methylation in cancer. It offers several advantages for detecting tumor DNA in the sera of patients with cancer. This technique is sensitive enough to detect even a very low level of tumor DNA in the serum as dilution experiments have shown that qMSP can detect approximately a single methylated gene copy among 1,000 unmethylated copies (18). It also has a high degree of specificity with no abnormal methylation in the serum being observed in cases where the corresponding tumors do not exhibit methylation (21). In our previous study of 44 patients with CRC, only four (9%) who exhibited *VIM* methylation in their tumors also exhibited methylation of *VIM* in their serum DNA (5). Moreover, no *VIM* methylation was detected in the serum of patients whose tumors were negative for *VIM* methylation (5). This observation supports the idea that qMSP could be clinically applicable for the detection of tumor DNA in the serum. Because *VIM* methylation of serum DNA is not specific to CRC and does not indicate that the colorectum is the primary tumor site, qMSP can also be used for detecting a wide variety of tumors that show aberrant methylation in gene promoters. In principle, the knowledge of the methylation status of a primary tumor is not required for detecting circulating tumor DNA, suggesting that qMSP can be used as a screening method for all types of cancer in general. However, the participants of this study are not representative of the general population. Future studies should, therefore, be conducted to evaluate an unselected screening population.

Conclusion

Our results suggest that *VIM* methylation may be a useful marker for detecting tumor DNA in the serum of patients with CRC. Additionally, the use of this method may improve the acceptance of CRC screening in the general population as the procedure can easily be integrated into a routine health check-up. The data obtained in this study may serve as the basis for developing blood-based assays for CRC screening.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

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