# Detection of Vimentin Methylation in the Serum of Patients with Gastric Cancer

ATSUSHI SHIRAHATA, KAZUMA SAKURABA, YOUHEI KITAMURA, KAZUAKI YOKOMIZO, TETSUROU GOTOU, MITSUO SAITOU, GAKU KIGAWA, HIROSHI NEMOTO, YUTAKA SANADA and KENJI HIBI

*Gastroenterological Surgery, Showa University Fujigaoka Hospital, Aoba-ku, Yokohama, Japan*

**Abstract.** *Aim: Detection of gastric cancer using serum assay of vimentin methylation. Methods: A quantitative methylation-specific polymerase chain reaction assay was used to detect vimentin gene (VIM) methylation in the serum of 71 patients with gastric cancer. Results: Mean VIM methylation in cancer patients (0.304±0.558) was significantly higher than that in healthy donors (0.011±0.015, p=0.018). The sensitivity of VIM methylation (33.8%) was similar to the one of carbohydrate antigen 19-9 (CA19-9) (25.4%), higher than the one of carcinoembryonic antigen (CEA) (12.7%), and significantly higher than the sensitivity of both markers for patients with stage I and IV disease (p=0.010 and 0.044, respectively). At all stages, the sensitivity of a combination of markers was higher than the sensitivity of any in isolation marker and was similar for stages I, II and III, reaching 76.9% for stage IV disease. Conclusion: VIM methylation may represent a useful marker for the detection of tumor DNA in the serum of patients with gastric cancer.*

Circulating DNA has been detected in the serum of cancer patients (1, 2). As a result, there have been many attempts to design an assay for the early detection of tumor-related aberrant DNA in the serum of patients with various malignancies (3, 4). In particular, we have detected tumor-specific DNA in the serum of patients with various types of cancer by using a mismatch ligation assay for *KRAS* and mitochondrial DNA mutations (5-8).

Promoter methylation has recently been established as an important mechanism for inactivating gene transcription. Several genes, including *p16* (9), *p14* (10), helicase-like transcription factor (*HLTF*) (11), suppressor of cytokine signaling-1 (*SOCS-1*) (12), and cadherin 13 (*CDH13*) (13), exhibit promoter hypermethylation associated with a loss of gene expression in digestive tract cancer. Therefore, the presence of epigenetic methylation may represent a useful molecular target for the detection of tumor DNA.

The methylation status of *p16* in colorectal cancer was previously examined using methylation-specific PCR (MSP) (14). We observed that DNA from 44 out of 94 tumors (47%) displayed abnormal promoter methylation of *p16*. Subsequently, we found that 13 out of 44 patients (30%) with *p16* promoter methylation of tumor DNA also demonstrated abnormal methylation of serum DNA. Thus, we aimed to develop a molecular biological technique to detect methylation of serum DNA.

The vimentin gene (*VIM*), usually activated in mesenchymal cells, was recently shown to be highly methylated in colorectal carcinoma (15). Indeed, *VIM* gene methylation was detected in 53%-84% of colorectal carcinomas (16-18). In addition, we detected aberrant methylation of *VIM* in 14 out of 37 primary gastric carcinomas (38%) (19). Therefore, gastric cancer might be detected and monitored by analyzing *VIM* methylation in clinical samples, such as serum and stool samples (20). In the present study, we aimed to detect *VIM* methylation in the serum of patients with gastric cancer.

## Materials and Methods

*Sample collection and DNA preparation.* The case-control study comprised 21 healthy donors and 71 patients with gastric cancer (excluding residual gastric cancer). Blood samples from patients with gastric cancer were obtained one week prior to surgery at Showa University Fujigaoka Hospital, Yokohama, Japan. In addition, blood samples from healthy donors were obtained at the hematology unit of the same hospital. Written informed consent, in accordance with the guidelines of the Institutional Review Board, was obtained from all patients. Serum was separated by centrifugation of peripheral blood at 2,500 rpm for 15 min, and was then immediately frozen and stored at −80˚C. For DNA extraction, the serum was digested with proteinase K, and the DNA was prepared as described previously (19). The clinicopathological profiles of patients enrolled in the study are presented in Table I. Normal levels of carcinoembryonic antigen (CEA) and carbohydrate

*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

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

| | | | *VIM* methylation in serum DNA | | |
|---|---|---|---|---|---|
| | | No. | Negative | Positive | *p*-Value |
| Age (years, mean±S.D.) | | 71 | 70.9±10.4 | 71.1±8.9 | 0.933‡ |
| Gender | Male | 52 | 35 | 17 | 0.743* |
| | Female | 19 | 12 | 7 | |
| CEA (ng/ml) | ≤5 | 62 | 40 | 22 | 0.432* |
| | >5 | 9 | 7 | 2 | |
| CA19-9 (U/ml) | ≤37 | 53 | 33 | 20 | 0.229* |
| | <37 | 18 | 14 | 4 | |
| Histology | Well differentiated | 24 | 14 | 10 | 0.317* |
| | Poorly differentiated | 47 | 33 | 14 | |
| Location | Upper region of the body | 17 | 12 | 5 | 0.304* |
| | Middle region of the body | 24 | 13 | 11 | |
| | Lower region of the body | 30 | 22 | 8 | |
| Size units | <45 | 20 | 13 | 7 | 0.937* |
| | ≥45 | 50 | 33 | 17 | |
| | Unknown | 1 | 1 | 0 | |
| Depth of tumor invasion | Tis, T1a, T1b | 12 | 8 | 4 | 0.347* |
| | T2 | 6 | 3 | 3 | |
| | T3 | 19 | 13 | 6 | |
| | T4a | 30 | 22 | 8 | |
| | T4b | 4 | 1 | 3 | |
| Lymph node metastasis | N0 | 29 | 18 | 11 | 0.556* |
| | N1 | 11 | 9 | 2 | |
| | N2 | 14 | 8 | 6 | |
| | N3a, N3b | 17 | 12 | 5 | |
| Peritoneal dissemination | No | 62 | 42 | 20 | 0.470* |
| | Yes | 9 | 5 | 4 | |
| Distant metastasis | M0 | 58 | 42 | 16 | 0.019* |
| | M1 | 13 | 5 | 8 | |
| TNM stage | IA, IB | 17 | 10 | 7 | 0.056* |
| | IIA, IIB | 16 | 13 | 3 | |
| | IIIA, IIIB, IIIC | 25 | 19 | 6 | |
| | IV | 13 | 5 | 8 | |
| Total | | 71 | 47 | 24 | |

*Chi-square test; ‡Student's *t*-test; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; TNM: tumor-node-metastasis.

antigen 19-9 (CA19-9) were defined as <5 ng/ml and <37 U/ml, respectively. The 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 established by the International Union Against Cancer (revised in 2009) (20).

*Sodium bisulfite modification*. Genomic DNA (1 μg) extracted from 200 μl of the corresponding serum were subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany).

*Quantitative methylation-specific polymerase chain reaction (qMSP)*. Bisulfite-treated DNA was amplified by 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 μl serum DNA sample, 100 nM each of the *VIM* or β-actin (*ACTβ*) primers (forward and reverse), and 12.5 μl SYBR Premix Ex *Taq* II (Takara Bio Inc.), which comprised *Taq* DNA polymerase, reaction buffer, and a deoxynucleotide triphosphate mixture. The qMSP primer sequences for *VIM* (15) were as follows: *VIM* MS (sense), 5'-TCGTTTCGAGGTTTTCGCGTTAGAGAC-3' and *VIM* MAS (antisense), 5'-CGACTAAAACTCGACCGACT CGCGA-3'. PCR amplification comprised an initial denaturation step (95˚C for 10 s) followed by 40 cycles (95˚C for 5 s and 55˚C for 30 s). Bisulfite-treated DNA that was obtained from L132 cells and fully methylated by *Sss*I methylase served as a positive control. To correct for differences in both the quality and quantity of DNA between samples, *ACTβ* 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 serum DNA (normalized to the internal control) was >0.2.

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

Table II. *Sensitivity of serum markers for gastric cancer according to TNM stage.*

| | TNM Stage | | | | |
|---|---|---|---|---|---|
| | I (n=17) | II (n=16) | III (n=25) | IV (n=13) | Total (n=71) |
| CEA | 0 (0%)* | 2 (12.5%) | 5 (20%) | 2 (15.4%)** | 9 (12.7%) |
| CA19-9 | 3 (17.6%)* | 3 (18.8%) | 8 (32%) | 4 (30.8%)** | 18 (25.4%) |
| *VIM* methylation | 7 (41.2%)* | 3 (18.8%) | 6 (24%) | 8 (61.5%)** | 24 (33.8%) |
| CEA + CA19-9 +*VIM* methylation | 10 (58.8%) | 7 (43.8%) | 15 (60%) | 10 (76.9%) | 42 (59.2%) |

*$p$=0.010; **$p$=0.044.

## Results

The mean level of *VIM* methylation in the DNA of 71 patients with gastric cancer was 0.304±0.558 (range, 0–2.79), whereas that of 21 healthy donors was 0.011±0.015 (range, 0-0.049). A significant difference between the two groups was observed ($p$=0.018). In addition, clinicopathological data were correlated with the results of the qMSP analysis (Table I). We found no association between serum levels of *VIM* methylation and age, gender, CEA level, CA19-9 level, histology, tumor location, tumor size, depth of tumor invasion, lymph node metastasis, or peritoneal dissemination. However, a significant difference was observed for distant metastasis between patients with *VIM* methylation and patients without ($p$=0.019). Moreover, a trend was observed for the TNM stage ($p$=0.056). Interestingly, *VIM* methylation was found in the serum of gastric patients at every stage, suggesting that the MSP technique may be used to detect gastric cancer even at the earliest stages.

We subsequently compared the sensitivity of the *VIM* methylation assay in the serum of gastric cancer patients with assays for CEA and CA19-9 markers (Table II). The sensitivity of *VIM* methylation as a tumor marker was 33.8%, which was similar to the one of CA19-9 (25.4%) and higher than the one of CEA (12.7%). Moreover, *VIM* methylation was a more sensitive marker than CEA or CA19-9 for patients with stage I or IV tumors (stage I, $p$=0.010; stage IV, $p$=0.044). We subsequently examined the sensitivity of a combination of all three serum markers for early detection of gastric cancer. For patients at every stage, the sensitivity of the combination of markers was higher than that of any marker in isolation (Table II). Moreover, while the sensitivity was similar for patients with stage I (58.8%), stage II (43.8%), and stage III disease (60.0%), the sensitivity was 76.9% for patients with stage IV disease (Table II).

## Discussion

Gastric cancer is one of the most common types of cancer worldwide, ranking fourth in overall frequency and accounting for over 650,000 deaths annually. Moreover, the mortality rate of gastric cancer is exceeded only by that of lung cancer (21). Treatment involves surgery combined with chemotherapy and radiotherapy. Therefore, it is important to identify the prevalence of genetic alterations which may represent new parameters to estimate the malignancy of gastric cancer.

Tumor-related aberrant DNA has been identified in the serum of cancer patients. Methylation of DNA in the serum, largely restricted to cytokines within CpG-rich sequences (CpG islands), is a common epigenetic change in human cancer and represents a new generation of cancer markers (11). In particular, methylation of the *p16* tumor suppressor gene in serum has been studied in various malignancies, including esophageal cancer, colorectal cancer, non-small cell lung cancer, liver cancer, and head and neck cancer (14, 22-26). Detection of *VIM* methylation in the serum has also been proposed as a minimally invasive assay to monitor colorectal cancer (27). *VIM* methylation was found in the serum of 48 out of 81 colorectal cancer patients with colorectal cancer (59%), with levels gradually increasing with advancing tumor stage (27). We previously used qMSP to show that 4 out of 44 patients with colorectal cancer (9%) displayed serum *VIM* methylation (28). Furthermore, a significant level of methylation was 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) (28). In the present study on gastric cancer, *VIM* methylation in the serum correlated with distant metastasis and TNM stage. Therefore, methylation of *VIM* in the serum may be important for digestive tract carcinogenesis.

MSP is a useful technique for the detection of promoter methylation in the serum of cancer patients. In particular, MSP is sensitive enough to detect even a very low level of tumor DNA in serum, dilution experiments have shown that it can be used to detect as few as 1 methylated gene copy in 1,000 unmethylated copies (29). In addition, MSP displays a high degree of specificity, and abnormal methylation in the serum has not yet been observed in cases where the corresponding tumors do not exhibit methylation (14). Moreover, since *VIM* methylation of serum DNA is not specific for colorectal cancer and does not provide any indication of the location of the primary tumor, qMSP may be used to detect a wide variety of tumors that display aberrant methylation of gene promoters.

In conclusion, *VIM* methylation may represent a useful marker for the detection of tumor DNA in the serum of patients with gastric carcinoma. Although the population used in this study was small and further studies are required, these results suggest that *VIM* methylation in serum may serve as a new marker to monitor and screen for gastric carcinoma.

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