Detection of TFPI2 Methylation in the Serum of Gastric Cancer Patients

KENJI HIBI, TETSUHIRO GOTO, ATSUSHI SHIRAHATA, MITSUO SAITO, GAKU KIGAWA, HIROSHI NEMOTO and YUTAKA SANADA

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

Abstract. Background: Methylation of tissue factor pathway inhibitor-2 (TFPI2) has been detected in the stool of colorectal cancer patients. Using quantitative methylation-specific polymerase chain reaction (qMSP), 39 out of 215 (18%) patients exhibited TFPI2 methylation in their serum DNA, suggesting that a significant number of methylated TFPI2 existed in colorectal cancer patients’ sera. Materials and Methods: Methylation status of the TFPI2 gene was examined in sera derived from 73 patients with gastric cancer using qMSP and the correlation between the methylation status and the clinicopathological findings was evaluated. Results: Out of 73 serum samples, 7 (10%) exhibited TFPI2 methylation in their serum DNA by qMSP, suggesting that TFPI2 methylation existed in the serum of gastric cancer patients. After completion of qMSP analysis of all specimens, clinicopathological data were correlated with the molecular analysis. TFPI2 methylation was significantly more frequently found in serum of patients with lymph node metastasis (p=0.0040) and distant metastasis (p=0.0115). Conclusion: In principle, knowledge of the methylation status of a primary tumor is not required in advance in order to be able to detect circulating tumor DNA. Therefore, qMSP could be used as a cancer screening method.

Previous studies have proposed that 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 previous studies have 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) harbor 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.

Recently, methylation of tissue factor pathway inhibitor-2 (TFPI2) was detected in the stool DNA of colorectal cancer patients (14), suggesting that TFPI2 methylation is a potential novel marker that can be used to detect colorectal cancer. It has been proven that TFPI2 is a potential tumor suppressor gene which is expressed and is unmethylated in colonic epithelium from cancer-free individuals. Therefore, TFPI2 methylation in stool and serum is cancer-specific and can be used to detect colorectal cancer. We examined whether TFPI2 methylation can be used as a molecular marker for colorectal cancer by its detection in patients’ sera by using quantitative methylation-specific polymerase chain reaction (qMSP) (15). The qMSP analysis showed that 39 out of 215 (18%) patients exhibited TFPI2 methylation in their serum DNA, suggesting that a significant number of methylated TFPI2 existed in colorectal cancer patients’ sera. These results encouraged us to investigate whether tumor-specific methylation of TFPI2 can be detected in serum DNA of gastric cancer patients using a molecular biological technique and thus be used as a diagnostic marker.

Materials and Methods

Sample collection and DNA preparation. Seventy-three serum samples were obtained at the time of surgery from 73 patients with primary gastric carcinoma at Showa University Fujigaoka Hospital,
Yokohama, Japan. Twenty control serum samples from healthy volunteers and patients without cancer were also obtained. The study was approved by the Institutional Review Board, and written informed consent was obtained from all patients. 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 (16).

Sodium bisulfite modification.

Two hundred microliters of the corresponding serum was subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany). Finally, 100 μl of modified DNA was obtained from 200 μl serum.

Quantitative methylation-specific PCR (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 modified serum DNA sample, 100 nM of each of the TFPI2 or β-actin 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. Before the sample analyses, we performed optimization of the serum DNA volume from 1-10 μl, and found that 2 μl of serum DNA was most effectively amplified by qMSP. The DNA yield was usually 500-2000 ng from 200 μl serum. Therefore, 2 μl of modified DNA was sufficient for the detection of methylated DNA. However, DNA is lost in the process of bisulfite modification. Hence, to confirm the quantity and quality of modified serum DNA, β-actin was used as an internal control. The qMSP primer sequences for TFPI2 have been described elsewhere (14); these were TFPI2 MS (sense), 5’-GTTCGGTGTTGGTAAGCGTTC-3’ and TFPI2 MAS (antisense), 5’-CATAAAACGAACACCCGGAACCG-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. qMSP was performed 5 times for each sample. If the TFPI2 amplification curve was observed along with a correct dissociation curve, TFPI2 methylation was considered to be positive.

Statistical analysis. The correlation between the TFPI2 methylation status of serum samples and the corresponding patients’ clinicopathological characteristics was evaluated. Statistical significance was evaluated by Chi-square test or Student’s t-test.

Results

We first examined the methylation status of TFPI2 in the serum DNA of gastric cancer patients. Out of 73 serum samples, 7 (10%) exhibited TFPI2 methylation in their serum DNA by qMSP, suggesting that TFPI2 methylation was present in DNA from the serum of gastric cancer patients. No methylation of TFPI2 was found in the serum DNA of 20 healthy volunteers and patients without cancer.

After completion of qMSP analysis of all specimens, clinicopathological data were correlated with the molecular
analysis (Table I). We found no association of overall methylation in the serum DNA with gender, age, size, histology, extent of tumor, hepatic metastasis, peritoneal dissemination, or stage. Interestingly, \textit{TFPI2} methylation was significantly more frequently found in serum DNA of patients with lymph node metastasis ($p=0.0040$), and distant metastasis ($p=0.0115$), suggesting that \textit{TFPI2} methylation in serum could be detected more easily in patients with advanced gastric cancer. The reason for this may be because the percentage of tumor DNA is usually higher in the sera of these patients. This does not mean that \textit{TFPI2} methylation is not present in earlier stages.

Discussion

In previous studies, tumor-related aberrant DNA has been identified in the serum of cancer patients. In particular, methylation of \textit{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 (17-21). 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. No abnormal methylation was found in serum DNA if this alteration was absent from the primary tumor. Moreover, MSP has sufficient sensitivity to detect even a very low level of tumor DNA in serum, being able to detect approximately 1 methylated gene copy/1000 unmethylated copies in dilution experiments (22).

qMSP has several advantages in detecting tumor DNA in the serum of cancer patients: (i) Its high sensitivity makes it possible to detect small amounts of tumor DNA in serum. (ii) This technique also has great specificity, and no abnormal methylation in serum has yet been observed if the corresponding tumors do not exhibit methylation. These observations support the clinical use of qMSP in the detection of tumor DNA in a patient’s serum. (iii) qMSP can be used to detect a wide variety of tumors that have shown aberrant methylation in gene promoters. (iv) In principle, knowledge of the methylation status of a primary tumor is not required in advance in order to be able to detect circulating tumor DNA. Therefore, qMSP could be used as a cancer screening method.

In this investigation, we used \textit{TFPI2} methylation as a target to detect tumor DNA in the serum of gastric cancer patients. The clinical sensitivity of this assay can potentially be improved by incorporating other possibly methylated targets such as \textit{p16}. It would be interesting to study the variation in circulating tumor DNA levels in relation to cancer treatment and patient prognosis.

Acknowledgements

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

References


