Abstract. Background, Materials and Methods: For the purpose of colorectal cancer detection, we investigated fibrillin-2 (FBN2) methylation in the serum of colorectal cancer patients using quantitative methylation-specific polymerase chain reaction (qMSP). Results: Out of 78 patients with colorectal cancer, 49 (63%) exhibited methylation of FBN2 in their tumor tissue DNA, suggesting that FBN2 methylation frequently exists in colorectal cancer. We next examined the methylation status of FBN2 in the serum DNA of patients with colorectal cancer. Out of 49 serum samples, four (8%) exhibited FBN2 methylation in their serum DNA by qMSP, suggesting that FBN2 methylation exists in the serum of colorectal cancer patients. After completion of qMSP analysis in all specimens, clinicopathological data were correlated with the molecular analysis findings. Interestingly, methylation of FBN2 was found in the serum DNA of male (p=0.0167) patients, and in those with hepatic metastasis (p<0.0001). Conclusions: The clinical sensitivity of this assay can be potentially improved by incorporating other common genetic targets such as p53 and KRAS. Advances in technology which will permit for rapid detection of an array of specific mutations and methylation would enhance the utility of this approach.

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 patients with various types of cancer using a mismatch ligation assay for Kirsten rat sarcoma viral oncogene homolog (KRAS) and mitochondrial DNA mutations (5-8).

Recent studies have indicated that promoter methylation is an important mechanism for inactivation of gene transcription. We and others have 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 polymerase chain reaction (MSP) (14). Forty-four out of 94 (47%) samples of tumor DNA exhibited abnormal promoter methylation of the p16 gene. Subsequently, we examined whether aberrant methylation could be detected in the corresponding serum DNA, and found that 13 out of 44 (30%) patients with p16 promoter methylation in their tumor DNA demonstrated abnormal methylation in their serum DNA as well. This result encouraged us in our attempts to detect methylation in serum DNA using a molecular biological technique.

FBN2 methylation has been detected in human non-small cell lung cancer (15). This report indicated that FBN2 methylation is a novel marker of cancer. In the current study, we first examined FBN2 methylation in colorectal cancer. Then, we tried to detect the FBN2 methylation in the serum DNA of patients with colorectal cancer.

Materials and Methods

Sample collection and DNA preparation. Tumor samples were obtained at the time of surgery, from 78 patients with primary colorectal carcinoma at the Showa University Fujigaoka Hospital, Yokohama, Japan. Out of these 78 patients, 24 (31%), 7 (9%), and 3 (4%) exhibited lymph node, hepatic, and distant metastasis, respectively. Seventy-eight corresponding serum samples had been obtained one 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 extraction. The samples were digested with proteinase K, and DNA was prepared as described previously (16).
Sodium bisulfite modification. One microgram of the genomic DNA extracted from the tumour specimens and 200 μl of the corresponding serum were subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany). Finally, 100 μl of modified DNA were 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 serum DNA sample, 100 nM of each of the FBN2 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 experiment, we performed the optimazation of the serum DNA volume from 1 μl to 10 μl. Two microliters of serum DNA were most effectively amplified by qMSP. The qMSP primer sequences for FBN2 have been described elsewhere (15) and were: FBN2 MS (sense): 5’-GGGAATTCGTCGAGTTTTGC-3’, and FBN2 MAS (antisense): 5’-AACCGACAACCCCGAACG-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). 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, β-actin was used as an internal control. The targets were obtained from the same bisulfite-treated DNA. qMSP was performed five times on each sample.

FBN2 methylation scores. The relative amounts of FBN2 methylated DNA in colorectal carcinomas and in corresponding normal tissues, that were normalised to the internal control β-actin, were calculated. The FBN2 methylation score in each tissue was defined as follows: relative amount of FBN2 in tumour/average relative amount of FBN2 in all corresponding normal tissues. FBN2 methylation was deemed to be positive when the methylation score was greater than 2.0.

Statistical analysis. We evaluated the correlation between the FBN2 methylation status of serum DNA samples and the clinicopathological characteristics of the patients. Statistical significance was evaluated by the Chi-square test or Student’s t-test.

Results

We first examined the methylation status of FBN2 in the tumor DNA of colorectal cancer patients. Out of 78 colorectal carcinomas, 49 (63%) exhibited methylation of FBN2, shown by qMSP, indicating that FBN2 methylation was frequently detected in colorectal cancer.

We next examined the methylation status of FBN2 in the serum DNA of the same patients. Out of 49 serum samples derived from patients with FBN2-positive colorectal cancers, four (8%) exhibited FBN2 methylation in their serum DNA by qMSP, suggesting that FBN2 methylation existed in the serum of colorectal cancer patients. More significantly, no
methylation of FBN2 was found in the serum DNA of 29 patients with colorectal cancer in whom the corresponding tumor DNA had no FBN2 methylation.

After completion of the qMSP analysis of all specimens, clinicopathological data were correlated with the molecular analysis (Table I). We found no association of overall methylation of FBN2 in the serum DNA with age, maximal tumor size, extent of tumor, tumor site, histology, presence of lymph node metastasis, distant metastasis, or Dukes’ stage. Interestingly, methylation was significantly found in the serum of male (p=0.0167) patients, and in those with hepatic metastasis (p<0.0001), suggesting that FBN2 methylation in serum may be more frequently detected in patients with colorectal cancer with hepatic metastasis.

Discussion

In previous studies, tumor-related aberrant DNA has been identified in the serum of patients with cancer. In particular, methylation of the p16 tumor-suppressor gene has been studied in various malignancies such as esophageal, colorectal, non-small cell lung, liver, and head and neck cancer (14, 17-21). For the detection of promoter methylation in these types of cancers, the MSP technique is a beneficial procedure because of its high sensitivity and specificity. No alterations could be detected in the serum by using KRAS mutations in the plasma of pancreatic cancer patients. Clin Cancer Res 4: 271-275, 1998.


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References


