Methylation of *TFPI2* No Longer Detected in the Serum DNA of Colorectal Cancer Patients after Curative Surgery

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Abstract. *In our previous study, we used quantitative methylation-specific polymerase chain reaction (qMSP) to examine the methylation status of tissue factor pathway inhibitor 2 (TFPI2) in the preoperative serum DNA of 215 colorectal cancer patients and found that TFPI2 was methylated in serum DNA from 39 of these patients. In this study, we examined postoperative serum DNA, obtained within one month after surgery from 38 out of the 39 patients and found that TFPI2 was methylated in the serum DNA of only 18 (47%) of these patients, suggesting that TFPI2 methylation in the serum of the remaining colorectal cancer patients was abolished by surgical tumor reduction. Next, we examined the correlation between the presence of TFPI2 methylation in postoperative serum DNA and residual cancer status after surgery. If R0 (no residual cancer) operations were successfully performed, TFPI2 methylation was not detected in postoperative serum. However, if R2 (obvious residual cancer) operations were performed, 17 (77%) out of 22 postoperative sera, still exhibited TFPI2 methylation. Taken together, our results confirm that detection of methylated TFPI2 in serum DNA was derived from colorectal cancer and could serve as a marker of surgical outcome.*

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 previous studies have shown that it is possible to detect tumor-specific DNA in the serum of various cancer patients by using a mismatch ligation assay for *KRAS* and mitochondrial DNA mutations (5-8).

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Recently, methylation of *tissue factor pathway inhibitor 2* (*TFPI2*) has been detected in the stools of colorectal cancer patients (9), suggesting that *TFPI2* methylation is a potential novel marker for the detection of colorectal cancer. Furthermore, *TFPI2* has been shown to be a potential tumor suppressor gene, which is expressed and is unmethylated in the colonic epithelium from cancer-free individuals. *TFPI2* methylation in stool and serum is cancer specific and can be used to detect colorectal cancer (9).

Therefore, we examined whether *TFPI2* methylation can be used as a molecular marker for colorectal cancer by detecting *TFPI2* methylation with quantitative methylation-specific polymerase chain reaction (qMSP) in the sera of colorectal cancer patients (10). The qMSP analysis showed that 39 out of 215 (18%) patients exhibited *TFPI2* methylation in their serum DNA, suggesting that *TFPI2* methylation occurred in the sera of colorectal cancer patients.

The aim of this study was to determine whether *TFPI2* methylation in serum is derived from colorectal cancer and could be used as a marker for determining surgical outcome. To address this, we examined the *TFPI2* methylation status in serum DNA of colorectal cancer patients after curative surgery.

Materials and Methods

Sample collection and DNA preparation. Preoperative serum samples were obtained at the time of surgery from 215 patients with primary colorectal carcinoma at Showa University Fujigaoka Hospital, Yokohama, Japan as described previously (10). The qMSP analysis showed that 39 out of the 215 (18%) serum DNA samples exhibited *TFPI2* methylation. Postoperative serum samples were obtained within one month after surgery from 38 out of the 39 patients. This study was approved by the Institutional Review Board, and written informed consent was obtained from all the patients. The 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 previously described (11).

Sodium bisulfite modification. Genomic DNA extracted from 200 μl of the serum was subjected to bisulfite treatment by using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany). A final volume of 100 μl of the modified DNA was obtained from the 200 μl serum.

Table I. *TFPI2 methylation status in serum DNA of colorectal cancer patients before and after surgery.*

Case no.	TNM stage	*TFPI2* methylation		Residual cancer after surgery
		Preoperative serum	Postoperative serum	
C024	3	+	–	R1[b]
C049	3	+	–	R0[a]
C061	4	+	–	R2[c]
C064	2	+	–	R0
C066	4	+	+	R2
C069	4	+	+	R2
C073	4	+	–	R2
C080	4	+	+	R2
C083	3	+	–	R0
C092	4	+	+	R2
C094	2	+	+	R1
C100	3	+	–	R0
C112	4	+	+	R2
C124	3	+	–	R0
C131	4	+	–	R2
C136	4	+	+	R2
C150	4	+	+	R2
C152	4	+	+	R2
C173	4	+	+	R2
C179	2	+	–	R0
C220	3	+	–	R0
C223	4	+	–	R2
C225	3	+	–	R0
C227	2	+	–	R0
C235	2	+	–	R0
C236	2	+	–	R0
C247	4	+	+	R2
C252	4	+	+	R2
C255	3	+	–	R0
C260	4	+	+	R2
C262	4	+	+	R2
C263	4	+	+	R2
C265	2	+	–	R0
C272	4	+	+	R2
C274	3	+	–	R0
C277	4	+	+	R2
C285	4	+	+	R2
C299	4	+	–	R2

[a]No residual cancer, [b]possible residual cancer, and [c]obvious residual cancer, detected after surgery according to the Japanese criteria (16).

qMSP. Bisulfite-treated DNA was amplified with qMSP by 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 each of the *TFPI2* or β-actin primers (forward and reverse), and 12.5 μl of SYBR Premix Ex *Taq* II (Takara Bio Inc.), which comprised Taq DNA polymerase, reaction buffer, and deoxynucleotide triphosphate mixture. Before the analysis, we performed optimization experiments with serum DNA volumes ranging from 1 to 10 μl, and found that 2 μl of serum DNA was most effectively amplified by qMSP. Most DNA yields usually ranged from 500 to 2000 ng from the initial 200 μl serum sample. Therefore, 2 μl of modified DNA was found to be sufficient for the detection of methylated DNA. However, some DNA was lost in the process of bisulfite modification. To confirm the quantity and quality of the modified serum DNA, β-actin was used as an internal control. The qMSP primer sequences for *TFPI2* have been described in another study (9) and are as follows: *TFPI2* MS (sense), 5'-GTTCGTTGGGTAAGGCGTTC-3' and *TFPI2* MAS (antisense), 5'-CATAAAACGAACACCCGAACCG-3'. The PCR amplification comprised 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, which was completely methylated using SssI methylase was used as a positive control. To correct for differences in both quality and quantity between the samples, β-actin was used as an internal control. The targets were obtained from the same bisulfite-treated DNA, and qMSP was performed five times per sample. If a *TFPI2* amplification curve was observed along with a correct dissociation curve, the serum sample was considered positive for *TFPI2* methylation.

Table II. *TFPI2 methylation status in serum DNA of colorectal cancer patients according to residual cancer after surgery.*

Residual cancer after surgery	No. of cases	*TFPI2* methylation	
		+	–
R0	14	0	14
R2	22	17	5
Total	36	17	19

Results

As previously described, we examined the *TFPI2* methylation status in preoperative serum DNA of colorectal cancer patients by using qMSP and found that 39 out of the 215 serum samples exhibited *TFPI2* methylation (10). No methylation of *TFPI2* was found in serum DNA of 20 healthy volunteers, patients without cancer, and four patients with colorectal cancer in whom the corresponding tumor DNA had no *TFPI2* methylation.

In this study, we examined postoperative serum DNA obtained within one month after surgery from 38 out of the 39 patients with positive *TFPI2* methylation identified in our previous study. Only 18 (47%) of the 38 serum samples, exhibited *TFPI2* methylation in their serum DNA, suggesting that surgical tumor reduction led to *TFPI2* methylation being abolished in the remaining colorectal cancer patients (Table I). Representative qMSP results are presented in Figure 1.

Finally, we examined the correlation between *TFPI2* methylation in postoperative serum DNA and the residual cancer status after surgery (Table II). If R0 (no residual cancer) operations were successfully performed, *TFPI2* methylation was found to be completely absent from the postoperative

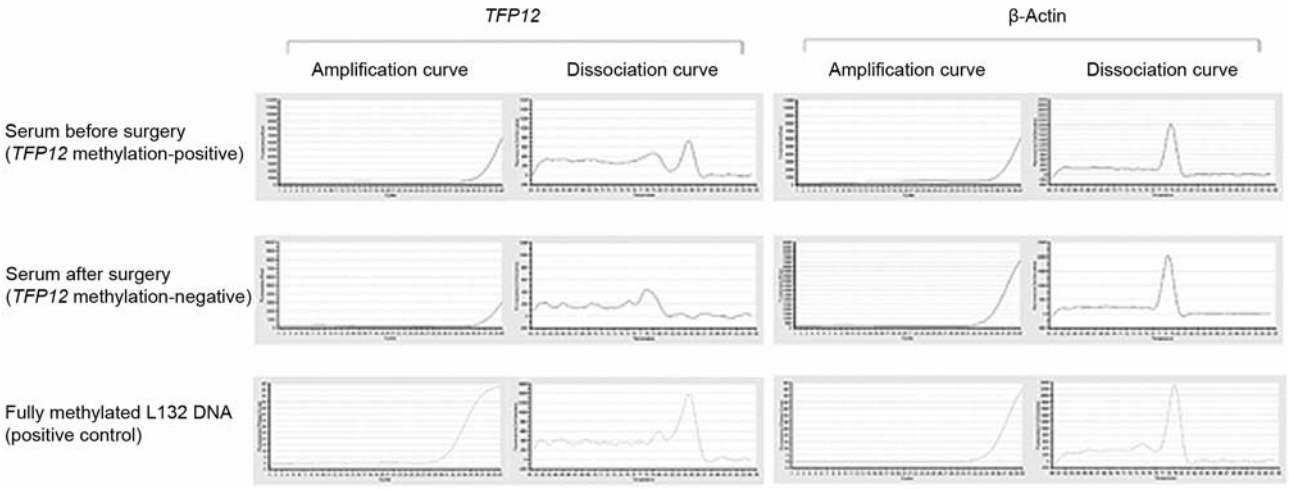

Figure 1. *Representative qMSP results (patient C083). If a TFPI2 amplification curve was observed along with a correct dissociation curve, we rendered the sample as being positive for TFPI2 methylation. Dissociation curve analysis confirming TFPI2-methylated DNA has a different melting temperature from the one of the specific product. Dissociation curve analysis was performed after complete PCR. Data was obtained by slowly ramping the temperature of reaction solutions from 60˚C to 95˚C while continuously collecting fluorescence data. The increase in temperature causes PCR products to undergo denaturation, a process accompanied by a decrease in fluorescence for solutions containing SYBR Green dye.*

serum of colorectal cancer patients. However, if R2 (obvious residual cancer) operations were performed, 17 (77%) out of 22 postoperative patients' sera still exhibited *TFPI2* methylation. Taken together, our results confirm that *TFPI2* methylation detected in serum DNA was derived from colorectal cancer and may be a marker of the surgical outcome.

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, including esophageal, colorectal, and head and neck cancer (12-14). For the detection of promoter methylation in these types of cancer, the MSP technique is advantageous 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 is sufficiently sensitive for the detection of a very low level of tumor DNA in serum, having previously detected as few as 1 methylated gene copy/1000 unmethylated copies in dilution experiments (15).

In this study, all 14 colorectal cancer patients who underwent R0 operations exhibited loss of *TFPI2* methylation in their serum DNA following surgery, suggesting that *TFPI2* methylation detected in their preoperative serum was indeed derived from colorectal cancer. Moreover, 17 out of 22 (77%) colorectal cancer patients who underwent R2 operations exhibited sustained presence of *TFPI2* methylation in their serum DNA after surgery, suggesting that *TFPI2* methylation in serum was derived from the residual cancer. However, 5 out of 22 (23%) colorectal cancer patients showed no *TFPI2* methylation in postoperative serum. Cancer volume reduction by surgery might be the reason why colorectal cancer patients who still have residual cancer exhibited no *TFPI2* methylation.

In this investigation, we used *TFPI2* methylation as a target to detect tumor DNA in the serum of colorectal cancer patients. qMSP has 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 serum. 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 cancer screening method.

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