Methylation of *TFPI2* Gene is Frequently Detected in Advanced Well-differentiated Colorectal Cancer

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Abstract. *Background: Recently, it has been reported that TFPI2 (tissue factor pathway inhibitor-2), a Kunitz-type serine proteinase inhibitor, is frequently methylated in human colorectal cancer using a gene expression array-based strategy. The aim of this study therefore was to examine whether the TFPI2 methylation in surgically removed colorectal cancers was correlated to the clinicopathological features. Materials and Methods: The methylation status of the TFPI2 gene was examined in primary carcinomas and corresponding normal tissues derived from 50 patients with colorectal cancer using quantitative methylation-specific PCR (qMSP), and the correlation between the methylation status and the clinicopathological findings was evaluated. Results. Methylation of the TFPI2 gene was detected in 31 out of the 50 (62%) primary colon carcinomas, suggesting that the methylation of TFPI2 is frequently observed in colorectal cancer. The clinicopathological data were compared with these results. Significant differences were observed between methylation of TFPI2 and histology (p=0.0053) or lymph node metastasis (p=0.0396). These results indicated that TFPI2 was more frequently methylated in well-differentiated advanced colorectal carcinomas. Conclusion: TFPI2 may act as a tumour suppressor in colorectal carcinomas and TFPI2 methylation may present a potential risk of malignancy in colorectal cancer.*

There is now firm evidence that a series of genetic alterations in both dominant oncogenes and tumour suppressor genes are involved in the pathogenesis of human colorectal cancer. The activation of oncogenes such as the *ras* gene, and the inactivation of tumour suppressor genes such as the *APC* (adenomatous polyposis coli) and *p53* genes, have been identified in colorectal cancer (1-3). In addition, it has also been found that several other genes are related to the pathogenesis of colorectal cancer (4, 5). An investigation of genetic changes is important in clarifying the tumorigenic pathway of colorectal cancer (6).

Recently, it has been reported that *TFPI2* (tissue factor pathway inhibitor-2) is frequently silenced in human hepatocellular carcinoma via epigenetic alterations, including promoter methylation and histone deacetylation (7). *TFPI2* is a Kunitz-type serine proteinase inhibitor that protects the extracellular matrix of cancer cells from degradation and inhibits *in vitro* colony formation and proliferation (7, 8). Subsequently, Glockner *et al.* demonstrated that the methylation of *TFPI2* is a frequent event in human colorectal cancer using a gene expression array-based strategy (9). These results therefore prompted the present study in which the methylation status of the *TFPI2* gene in surgically removed colorectal cancers was examined.

In the present study, the methylation status of the *TFPI2* gene was examined in primary carcinomas and the corresponding normal tissues derived from 50 patients with colorectal cancer and a comparison between the methylation status and the clinicopathological findings was performed.

Materials and Methods

Sample collection and DNA preparation. Fifty primary tumour and corresponding normal tissue specimens were collected consecutively at Showa University Fujigaoka Hospital from colorectal cancer patients during colorectal surgery. The status of all tissue specimens were confirmed histologically. Written informed consent, as required by the Institutional Review Board, was obtained from all the patients. The samples were stored immediately at –80˚C until analysis. The DNA was prepared as described elsewhere (10). The clinicopathological profiles of the patients enrolled in the study are shown in Table I.

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Table I. *Clinicopathological features and TFPI2 methylation in colorectal cancer.*

Clinicopathological feature	Variable	No. of cases	*TFPI2* methylation		*p*-Value
			+	–	
Gender	Male	26	18	8	0.272[1]
	Female	24	13	11	
Age (years)	45-87		66.7±9.8[3]	61.3±16.9	0.157[2]
Maximal tumour size (mm)	15-120		50.9±4.4[3]	40.6±5.8	0.166[2]
Extent of tumour	≤mt	12	7	5	0.765[1]
	mt<	38	24	14	
Tumour site	C, A, T	22	15	7	0.423[1]
	D, S, R	28	16	12	
Histology	Well	33	25	8	0.0053[1]
	Others[4]	17	6	11	
Lymph node metastasis	+	25	12	13	0.0396[1]
	–	25	19	6	
Duke's stage	A	6	5	1	0.222[1]
	B	17	12	5	
	C	27	14	13	
Total		50	31	19	

[1]Chi-square test; [2]Student's *t*-test; [3]mean±S.D; mt, muscular tunic; C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum; Well: well-differentiated adenocarcinoma according to Japanese criteria; [4]moderately- or poorly-differentiated, or signet ring cell adenocarcinoma according to Japanese criteria (15).

Sodium bisulfite modification. One μg of the genomic DNA extracted from the tumour and the corresponding normal colorectal tissue specimens was subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany).

Quantitative methylation-specific PCR (qMSP). The bisulfite-treated DNA was amplified with quantitative methylation-specific PCR (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 1.0 μL of the 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. The qMSP primer sequences for *TFPI2* have been described elsewhere (9) and were: *TFPI2* MS (sense), 5'-GTTCGTTGGGTAAGGCGTTC-3', and *TFPI2* MAS (antisense), 5'-CATAAAACGAACACCCGAACCG-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. 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.

TFPI2 methylation scores. The relative amounts of *TFPI2* methylated DNA in the colorectal carcinomas and the corresponding normal tissues that were normalized to the internal control β-actin were calculated. The *TFPI2* methylation score in each tissue was defined as follows: relative amount of *TFPI2* in tumour/relative amount of *TFPI2* in corresponding normal tissue.

swTFPI2 methylation was deemed to be positive when the methylation score was greater than 5.0.

Statistical analysis. The association between TFPI2 methylation and clinicopathological parameters was analysed using Chi-square tests or Student's *t*-tests. A p-value <0.05 indicated statistical significance. Statistical analysis was performed using statistical software, JMP8.

Results

Methylation of the *TFPI2* gene was detected in 31 out of the 50 (62%) primary colon carcinomas, suggesting that the aberrant methylation of *TFPI2* was frequently observed in colorectal carcinomas.

The clinicopathological data were compared with the methylation results. No significant differences were observed between the presentation of methylation in the colorectal carcinomas and patient gender or age, maximal tumour size, tumour extent, tumour site, or Dukes' tage (Table I). Significant difference was observed in the histology (p=0.0053) and lymph node metastasis (p=0.0396) (Table I). These results indicated that *TFPI2* was more frequently methylated in well-differentiated advanced colorectal carcinomas.

Discussion

Colorectal cancer is one of the most aggressive malignancies and occurs at a high incidence in most countries (11). One

treatment of this fatal cancer is surgery and subsequent chemotherapy and radiotherapy. For this purpose, it is important to identify the occurrence of genetic alterations as a new parameter to estimate the malignancy of the cancer.

In the present study, frequent methylation of *TFPI2* was observed in colorectal cancer. A significant increase was observed in the lymph node metastasis in the methylated *TFPI2* tumours. Moreover, well-differentiated colorectal carcinomas significantly showed the *TFPI2* methylation. The methylation status of the *p16* and *p14* genes in 86 primary colorectal carcinomas was examined previously by our group using MSP and a significant difference in maximal tumour size (p=0.022) was found when patients with both *p16* and *p14* methylation were compared to other patients (12). The methylation status of the *HACE1* gene was also investigated and a significant increase was observed in the maximal tumour size of the methylated *HACE1* tumours (p=0.0304) (13). Moreover, a trend was shown towards a preferential development of lymph node metastasis in the methylated *HACE1* carcinomas (p=0.0612) (13). Furthermore, the methylation of the *UNC5C* gene was examined and a significantly greater proportion of cases with methylated UNC5C was found in Dukes' stage C (p=0.0380) than in earlier stages. (14). Taking all these results together, parameters such as large tumour size, lymph node metastasis, and advanced clinical stage indicated that the methylated status of colorectal carcinomas was significantly correlated with malignant potential.

This study provides firm evidence that can be used in further studies of the molecular mechanism of *TFPI2* in colorectal cancer. This study also suggests that *TFPI2* may play a role in the carcinogenic pathway in some patients with colorectal cancer. These observations indicate the possibility that tumour formation in the colorectum may thus be controlled by inducing the expression of silenced *TFPI2* using demethylating reagents.

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