Methylation of the *TFPI2* Gene Is Frequently Detected in Advanced Gastric Carcinoma

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Abstract. *Background: Recently, Glockner et al. identified the methylation of TFPI2 as a frequent event in human colorectal cancer using a gene expression array-based strategy. Materials and Methods: Methylation status of the TFPI2 gene was examined in primary carcinomas and the corresponding normal tissues derived from 38 patients with gastric cancer using quantitative methylation-specific PCR (qMSP) and the correlation between the methylation status and the clinicopathological findings was evaluated. Results: Aberrant methylation of the TFPI2 gene was detected in 7 out of 38 (18%) primary gastric carcinomas, suggesting that the methylation of TFPI2 is frequently observed in gastric carcinomas. The clinicopathological data were correlated with the methylation results. A significant difference was observed in maximal tumour size (p=0.0084), extent of tumour (p=0.0068), and TNM stage (p=0.0392). Conclusion: TFPI2 is frequently methylated in advanced gastric carcinomas.*

Accumulating evidence indicates that gastric cancer is the result of various genetic and epigenetic alterations of oncogenes, tumour suppressor genes, DNA repair genes, cell-cycle regulators, and cell adhesion molecules (1). Aberrant methylation of CpG-rich sequences (CpG islands) is an epigenetic change that is common in human cancer (2). In gastric cancer, the inactivation of human mutL homolog 1 (*hMLH1*), *O*-6-methylguanine-DNA methyltransferase (*MGMT*), tissue inhibitor of metalloproteinase 3 (*TIMP-3*) and *p16* by promoter hypermethylation has been demonstrated (3-6). There has been substantial interest in attempting to adapt such cancer-associated aberrant gene methylation for clinical use.

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Key Words: TFPI2, quantitative methylation-specific PCR, gastric cancer.

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*. identified the methylation of *TFPI2* as a frequent event in human colorectal cancer using a gene expression array-based strategy (9). These results prompted the present study to examine the methylation status of the *TFPI2* gene in gastric tumours surgically removed.

In the present study, the methylation status of the *TFPI2* gene was examined in primary carcinomas and the corresponding normal tissues derived from 38 patients with gastric carcinomas, and the correlation between the methylation status and the clinicopathological findings was evaluated.

Materials and Methods

Sample collection and DNA preparation. Thirty-eight primary tumour and corresponding normal tissue specimens were collected consecutively at Showa University Fujigaoka Hospital from gastric cancer patients during gastric surgery. All tissue specimens were confirmed histologically. Written informed consent, as required by the Institutional Review Board, was obtained from all patients. The samples were stored immediately at –80˚C until analysis. DNA was prepared as described elsewhere (10). Briefly, tumour samples were digested overnight by proteinase K and treated by phenol/chloroform. DNA was precipitated by ethanol and ammonium acetate. The clinicopathological profiles of the patients enrolled in the study are shown in Table I.

Sodium bisulfite modification. One µg of the genomic DNA extracted from the tumour and the corresponding normal gastric 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 qMSP conducted in 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 each of the *TFPI2* or *β-actin*

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

Clinicopathological feature	Variable	No. of cases	TFPI2 methylation		*p*-Value
			+	–	
Gender	Male	31	6	25	0.749†
	Female	7	1	6	
Age (years)	50-82	38	74.3±3.5*	68.2±9.3*	0.128‡
Maximal tumour size (mm)	10-130	38	82.9±32.0*	55.8±21.0*	0.0084‡
Extent of tumour	≤ss	14	0	14	0.0068†
	>ss	24	7	17	
Histology	Well	15	2	13	0.507†
	Poor	23	5	18	
Lymph node metastasis	+	24	5	19	0.610†
	–	14	2	12	
TNM stage	1	15	0	9	0.0392†
	2-4	23	7	22	
Total		38	7	31	

†Chi-square test; ‡Student's *t*-test; *mean±S.D; ss, subserosa; Well, well- or moderately-differentiated adenocarcinoma according to Japanese criteria; Poor, poorly-differentiated, mucinous, or signet ring cell adenocarcinoma according to Japanese criteria.

primers (forward and reverse) and 12.5 μl of SYBR Premix Ex Taq II (Takara Bio Inc.), which consists of Taq DNA polymerase, reaction buffer and deoxynucleotide triphosphate mixture. The qPCR primer sequences for *TFPI2* have been described elsewhere and were: *TFPI2* MS (sense), 5'-GTTCGTTGGGTAAGGCGTTC-3', and *TFPI2* MAS (antisense), 5'-CATAAAACGAACACCCGA ACCG-3'. 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, which were 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 gastric carcinomas and the corresponding normal tissues normalised 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/average relative amount of *TFPI2* in all corresponding normal tissues. *TFPI2* methylation was considered positive when the methylation score was more than 5.0.

Statistical analysis. The associations between *TFPI2* methylation and clinicopathological parameters were analysed using Chi-square tests or Student's *t*-tests. A *p*-value <0.05 indicated statistical significance.

Results

Methylation of the *TFPI2* gene was detected in 7 out of the 38 (18%) primary gastric carcinomas, suggesting that the methylation of *TFPI2* is frequently observed in gastric carcinomas.

The clinicopathological data were correlated with the methylation results. No significant correlations were observed between the presentation of methylation in gastric carcinomas and patient gender or age, histology, or lymph node metastasis (Table I). A significant difference was observed in maximal tumour size (p=0.0084), extent of tumor (p=0.0068) and TNM stage (p=0.0392) (Table I). These results indicated that *TFPI2* is frequently methylated in advanced gastric carcinomas.

Discussion

Gastric cancer is one of the most common malignancies worldwide (11). In order to treat this fatal cancer, surgical operations and subsequent chemotherapy and radiotherapy are performed. 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 gastric cancer. Moreover, a significant difference was observed in regard to maximal tumour size, extent of tumour and TNM stage, thus indicating that *TFPI2* is frequently methylated in advanced gastric carcinomas. Previously, the methylation status of the *MGMT* gene in primary carcinomas and the corresponding normal tissues derived from 38 patients with gastric cancer were examined using qMSP (12). Aberrant methylation of the *MGMT* gene was detected in 4 out of the 38 (11%) primary gastric carcinomas. A significant difference was observed in the extent of tumour (p=0.0470), lymph node metastasis (p=0.0470) and TNM stage (p=0.0377), suggesting that *MGMT* is frequently methylated in advanced gastric carcinomas. Taken together, parameters such as the extent of tumour and TNM stage indicated that the methylated status of gastric carcinomas is significantly correlated with

malignant potential. *TFPI2* methylation could be used as a tumuor marker in clinical samples such as serum, for the detection of gastric carcinomas (13-15).

This study provides a solid basis for additional studies on the molecular mechanism of TFPI2 in gastric carcinoma and the present findings also suggest that TFPI2 may play a role in the carcinogenic pathway in some patients with gastric carcinomas. Since gastric carcinoma is one of the most aggressive of all cancer types, it may not be possible to improve the overall survival rate using information about *TFPI2* methylation alone. However, *TFPI2* methylation could prove useful as a marker for advanced gastric carcinoma. *TFPI2* methylation should therefore be the target of future therapies for gastric carcinomas.

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Received April 14, 2010
Revised May 25, 2010
Accepted June 3, 2010