Diagnostic utility of aberrant methylation of tissue factor pathway inhibitor 2 in pure pancreatic juice for pancreatic carcinoma

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Diagnostic utility of aberrant methylation of tissue factor pathway inhibitor 2 in pure pancreatic juice for pancreatic carcinoma

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The tissue factor pathway inhibitor 2 (TFPI-2) is a Kunitz-type serine proteinase inhibitor. Recently, the aberrant methylation of *TFPI-2* was detected frequently in pancreatic carcinoma (PCa) tissues but not in normal pancreatic tissues. We analyzed the aberrant methylation of *TFPI-2* in the pure pancreatic juice (PPJ) aspirated endoscopically from patients with various pancreatic diseases. Using the highly sensitive methylation-specific polymerase chain reaction (MSP) and quantitative MSP (Q-MSP) assay, we investigated the aberrant methylation of *TFPI-2* in nine human PCa cell lines and in the PPJ from patients with PCa, intraductal papillary mucinous neoplasms (IPMN) and chronic pancreatitis (CP). The incidence of aberrant *TFPI-2* methylation was seven (77.8%) of nine PCa cell lines by Q-MSP. In cell lines, the expression of TFPI-2 mRNA by quantitative reverse transcription–polymerase chain reaction showed an inverse correlation to the aberrant methylation of *TFPI-2*. The incidence of aberrant *TFPI-2* methylation in the PPJ was 21 (58.3%) of 36 PCa patients, three (17.6%) of 17 IPMN and one (4.8%) of 21 CP by MSP assay. Using a suitable cut-off value of 2.5 according to the receiver operating characteristic curve, the incidence of aberrant *TFPI-2* methylation in the PPJ by real-time MSP was 18 (62.1%) of 29 PCa patients, one (5.1%) of 17 IPMN and three (14.3%) of 21 CP, respectively. The incidence of quantitative *TFPI-2* hypermethylation in the PPJ with PCa was significantly higher than that with IPMN ($P < 0.001$) or CP ($P < 0.001$). Moreover, the aberrant methylation rate of *TFPI-2* in the PPJ was 100%, as observed (6/6) in the PCa patients with liver metastasis, and 86.7% (26/30) in stages IVa + IVb of PCa by Q-MSP assay. These results suggest that promoter methylation of *TFPI-2* in the PPJ may be a useful marker in the diagnosis and progression of PCa using an endoscopically feasible approach. (*Cancer Sci* 2006; 97: 1267–1273)

Pancreatic ductal adenocarcinoma has a poor prognosis with an overall 5-year survival rate ranging from 0.4%[1] to 4%[2]; it is one of the top 10 causes of cancer death in the industrialized world.[3,4] Even in patients with PCa that underwent curative surgical resection, the 5-year survival rate was 21% among patients who received chemotherapy and 8% among patients who did not receive chemotherapy.[5] This is due both to the inherently aggressive biology of the disease and to its late diagnosis in most cases. Today, the diagnostic ability for fairly large PCa has improved markedly.[6] However, the number of patients with radical resection of PCa is still small and there are many cases in which differentiating a malignant lesion of the pancreas from a benign one is difficult. Although clinical doctors are able to use ultrasound sonography, computed tomography and endoscopic retrograde cholangio-pancreatography to characterize early pancreatic cancer from CP and IPMN, the diagnostic specificity is not sufficient to differentiate benign and malignant pancreatic tumors when the pancreatic tumor is small. Difficulty in obtaining a biopsy specimen in PCa, unlike in gastrointestinal cancer, and the low diagnostic ability in the cytology of pancreatic juice may be major factors for such diagnostic difficulty. Recently, the detection of specific tumor markers in PPJ has become attractive in the diagnosis of pancreatic diseases.

Based on information obtained from recent molecular biological studies on carcinogenesis, the activation of oncogenes or loss of function of tumor suppressor genes (a part of which is due to aberrant methylation) has been clarified to participate in tumor development and progression.[6,7] The oncogene K-*ras* and tumor suppressor gene *p53* have been researched widely all over the world.[8,9] Although the oncogene and tumor suppressor gene play a critical role in tumor diagnosis, they are insufficiently specific or sensitive to improve the diagnosis of PCa. Recently, aberrant methylations of tumor-related genes are attractive in the tumor marker investigation. In human cancers, CpG island methylation occurs aberrantly and is associated with the inappropriate silencing of tumor suppressor genes and other genes that function in the suppression of malignant phenotypes.[10,11] Methylation-mediated silencing contributes to malignant progression and has been implicated in the inactivation of genes involved in tumor suppression.

The molecular markers in human PPJ are thought to be accurate predictors of the early diagnosis of pancreatic disease because PPJ has a high concentration of DNA, RNA and protein released from PCa compared with other clinical sources, such as the serum. We have previously reported the usefulness of analyzing K-*ras* and *p53* mutations in the supernatant and sediment of PPJ for the diagnosis of PCa.[12–21] The frequency of K-*ras* mutations in PCa tissue ranges from 70% to almost 100%. The incidence of KRM in PPJ from patients with PCa was over 80% using a sensitive method, such as mutant allele-specific amplification. However, KRM was observed in 28% of PPJ from the patients with CP. Many other studies also reported the frequent presence (20–30%) of KRM in PPJ from non-cancerous diseases with conditions such as CP and IPMN, raising questions as to the cancer specificity of this marker. A qualitative analysis of KRM in PPJ is unsuitable for definite diagnosis of PCa but is appropriate for PCa screening. In contrast, a hybridization protection assay using acridinium ester-labeled DNA probes can quantitatively determine the KRM in PPJ. Using this method, quantitative analysis of KRM in PPJ may be useful for differentiating PCa from CP by using a suitable cut-off value. Although

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Abbreviations: CP, chronic pancreatitis; ECM, extracellular matrix; IPMN, intraductal papillary mucinous neoplasm; KRM, K-*ras* mutation at codon 12; MMP, matrix metalloproteinase; MSP, methylation-specific PCR; PCa, pancreatic adenocarcinoma; PCR, polymerase chain reaction; PPJ, pure pancreatic juice; Q-MSP, quantitative methylation-specific PCR; RT, reverse transcription; PCR-SSCP, PCR-single strand conformation polymorphism; TFPI-2, tissue factor pathway inhibitor 2; Tm, melting temperature.

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the frequency of *p53* mutations has been reported to be 40–76% in PCa tissues, in our previous report, *p53* mutations were found in 42% of PPJ sediment from the patients of PCa examined by PCR-SSCP and direct sequencing, but were not detectable in PPJ from 16 CP and four adenoma patients, suggesting that specificity of *p53* mutation is very high for PCa. However, the sensitivity of *p53* mutation in PPJ was not so high. Therefore, such genetic analyses seem to be limited by relatively high false positives or insufficient sensitivity. Therefore, a new reliable marker is needed for the diagnosis of PCa.

Tissue factor pathway inhibitor-2, a structural analog of TFPI, is a 32-kDa Kunitz-type serine proteinase inhibitor that inhibits plasmin, trypsin, chymotrypsin, cathepsin G and plasma kallikrein, and has also been shown to reduce tumor invasion and metastasis.[22–26] The human *TFPI-2* gene is located on chromosome 7q22 and its promoter contains a CpG island region of 220 bp that spans exon 1 and three transcription initiation sites. Recombinant TFPI-2 inhibits the plasmin-mediated matrigel and the degradation of ECM in a dose-dependent manner.[23] The amino terminal sequence and molecular cloning of the cDNA revealed that TFPI-2 is identical to retinal pigment epithelial cell factor-1 and placental protein 5.[24] TFPI-2 is expressed abundantly in full-term placenta and widely in a variety of adult human tissues such as liver, skeletal, muscle, heart, kidney and pancreas.[25] TFPI-2 effectively decreases the activation of MMP-1, MMP-3 and MMP-9, and reduces the invasive potential of several tumor cells including HT-1080 fibrosarcoma cells and urokinase-charged Hela cells.[26] TFPI-2 mRNA was induced after demethylation and histone deacetylation inhibition treatment *in vitro*.[27,28] Aberrant methylation has been suggested as a possible mechanism for the loss of TFPI-2 expression in tumor cell lines. Steiner *et al*. reported that 5-Aza 2′-deoxycytidine/depsipeptide FK228 treatment induced TFPI-2 expression in CALU-6 and H460 lung cancer cells.[27] *TFPI-2* promoter methylation was observed in 20% (1/5) of pulmonary adenocarcinomas, and 100% (7/7) of esophageal adenocarcinomas. Sato *et al*. reported that aberrant methylation of *TFPI-2* was detected in 73% of PCa xenografts and primary PCa.[29] Hypermethylation of *TFPI-2* was also detected in 57% of the PPJ obtained at surgery from PCa patients and none of the benign pancreatic disorder patients by MSP assay.

Previous studies implied that *TFPI-2* is frequently silenced in carcinoma because of hypermethylation and deacetylation. The aim of the present study was to analyze the aberrant methylation of *TFPI-2* in PPJ aspirated by an endoscopically feasible approach from patients with various pancreatic disorders and to evaluate its tumor marker value in the diagnosis and prognosis of PCa.

Materials and Methods

Pancreatic caner cell lines. Nine human pancreatic cancer cell lines were used in this research. The six human PCa cell lines (KLM-1, PK-45H, PK-59, PK-1, PK-8 and PK-9) were provided by the Cell Resource Center for Biomedical Research Institute of Development Aging and Cancer Bank, Tohoku University (Sendai, Japan). The human PCa cell line Mia-PaCa-2 was provided by the Cell Bank at the Riken Bioresource Center (Tsukuba, Ibaragi, Japan). The two human PCa cell lines PANC-1 and BxPC-3 were purchased from Dainippon Pharmaceutical Company (Osaka, Japan). PANC-1 and Mia-PaCa-2 were cultured and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin G (100 U/mL) and kanamycin sulfate (100 μg/mL). KLM-1, PK-45H, PK-59, PK-1, PK-8, PK-9 and BxPC-3 cells were cultured and maintained in RPMI-1640 with 10% fetal bovine serum, penicillin and kanamycin at 37°C in a 5% CO_2, 95% air atmosphere.

Human pancreatic diseases samples. The PPJ samples from 36 patients with PCa, 10 with malignant IPMN, seven with benign IPMN and 21 with CP were investigated in the present study (Table 1). The patients were diagnosed and treated at the Department of Internal Medicine, Cancer Research Institute Hospital, Kanazawa University and Kanazawa University Hospital, and its affiliated facilities in Japan from 1993 to 2005. Informed consent for this study was obtained from all of the patients. Ultrasound sonography, computed tomography and endoscopic retrograde cholangio-pancreatography were carried out in all cases. Diagnosis of the disease was confirmed based on the results of these modalities and laboratory data, including elevation of serum tumor markers, such as CA19-9 and CEA. Twenty PCa tumors were confirmed histologically at the time of operation or biopsy under endoscopic ultrasonography. The remaining 16 pancreatic tumors, which were not examined histologically, showed findings compatible with PCa on ultrasound sonography, computed tomography and endoscopic retrograde cholangio-pancreatography, and these patients died of PCa within 6–18 months of diagnosis with a compatible clinical course. As shown in Table 1, the mean ages of the PCa, malignant IPMN, benign IPMN and CP groups were 62.7 ± 12.3 years, 68.5 ± 8.2 years, 69.3 ± 6.6 years and 58.7 ± 13.3 years, respectively. There was no significant difference in age between the two groups. The staging of PCa was determined according to various diagnostic modalities or by operation according to the TNM Classification of Pancreatic Carcinoma as set out by the International Union Against Cancer.[30] The diagnosis of IPMN was made according to the classification of the Armed Forces Institute of Pathology.[31]

Table 1. Characterization of enrolled patients with pancreatic diseases

Disease	Total no. cases	Mean age (years)	Sex	
			male	Female
PCa	36	62.7 ± 12.3	28	8
Malignant IPMN	10	68.5 ± 8.2	8	2
Benign IPMN	7	69.3 ± 6.6	5	2
CP	21	58.7 ± 13.3	15	6

CP, chronic pancreatitis; IPMN, intraductal papillary mucinous neoplasm; PCa, pancreatic adenocarcinoma.

The patients with IPMN were divided into two groups: a malignant group and a benign group. Clinically tentative definitions of malignant IPMN were required for at least one of three conditions under various modalities described below: (1) diameter of the main pancreatic duct more than 7 mm; (2) size of the cystic lesions more than 30 mm; (3) mural nodule more than 6 mm in size; or (4) borderline lesion or carcinoma histologically confirmed with operative materials. However, the benign IPMN did not satisfy any condition described above or show hyperplasia and adenoma was confirmed histologically with surgical materials. Finally, 10 patients were judged as malignant IPMN (including two with operation) and seven as benign IPMN (including one with operation). The diagnosis of CP was based on the clinical diagnostic curative of the Japan Pancreas Society.[32] The 21 patients with CP were classified as definite based on the clinical diagnostic curative and were also followed up over a 12-month period with no clinical detection of the PCa tumor.

Pure pancreatic juice samples. Pure pancreatic juice was collected using a duodenal endoscope (JF-10, JF-230, JF-240; Olympus, Tokyo, Japan) in the fasting state through a cannula inserted into the orifice of the papilla vater under stimulation with intravenous secretin (1 unit/kg), as described previously,[8] and stored at −80°C until use for genetic analysis.

DNA extraction. DNA extraction of PPJ, PCa cell lines, and primary PCa tissues was carried out using standard methods with a phenol–chloroform mixture, and precipitated with ethanol.

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RNA isolation and real-time RT-PCR. Total RNA was extracted from PCa cells using a SV Total RNA Isolation System kit (Promega, Madison, WI, USA). RNA concentrations were determined using spectrophotometry. RT was carried out using a PowerScript Reverse Transcriptase kit (Clontech Laboratories, Palo Alto, CA, USA). First-strand cDNA was synthesized from 8 μg of total RNA at 65°C for 5 min after RT; incubation at 42°C for 60 min activated the reverse transcriptase and the reaction was terminated with heating at 70°C for 25 min. The expression of TFPI-2 mRNA in the PC cell lines was carried out using the LightCycler real-time PCR apparatus (Roche Diagnostics, Mannheim, Germany) and LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics), as described by the manufacturer. The primers of *TFPI-2* for RT-PCR were 5′-CCAGATGAAGCTACTTGTATG-3′ for the sense primer and 5′-GCACATGCACGTTTGCAATC-3′ for the antisense primer, in accordance with a previous report.[29] The conditions for the real-time RT-PCR reactions were denaturation at 95°C for 10 s, annealing at 63°C for 8 s and elongation at 72°C for 15 s. Melting curves were obtained using the LightCycler 3.5 program. Samples with a similar Tm to the standard BxPC-3 cell line cDNA were judged as a positive result for TFPI-2 mRNA. 18S was used as an internal control.

Bisulfite modification and MSP. DNA samples were treated with sodium bisulfite for 16 h at 50°C and purified using a CpGenome DNA modification kit (Chemicon International, Temecula, CA, USA). MSP primers for *TFPI-2* were designed as: unmethylation forward, 5′-GGATGTTTGTTTTGTATAAAGTG-3′, and reverse, 5′-AAACATCCAAAAAAACACCTAAC-3′, producing an 89-bp fragment; methylation forward, 5′-TTTCGTATAAAGCGGG TATTC-3′, and reverse, 5′-ACGACCCGCTAAACAAAACG-3′, producing a 95-bp fragment.[29] Each MSP reaction incorporated 1 μg of bisulfite-treated DNA as a template, 10 pmol/L of each primer, 100 pmol/L deoxynucleoside triphosphate, 10× PCR buffer, and 1 unit of *Taq* polymerase in a final reaction volume of 20 μL. The cycle conditions were as follows: (1) 95°C for 5 min; (2) 35 cycles of 95°C for 20 s, 58°C for 20 s, and 72°C for 30 s; and (3) 72°C for 4 min. Aliquots (8 μL) of MSP products were analyzed with 3% agarose gel electrophoresis and visualized with SYBR Gold (Molecular Probes, Eugene, OR, USA) at a 10 000× dilution in dimethylsulfoxide, and exposed to ultraviolet 312-nm light.

Real-time Q-MSP. Bisulfite-modified DNA (2 μL) was used for fluorescence-based Q-MSP using the LightCycler real-time PCR apparatus, and Light Cycler Fast Start DNA Master SYBR Green I was used as described by the manufacturer. The methylation-specific primers for *TFPI-2* were used in the same way as for the MSP assay. The conditions for the Q-MSP reactions were: denaturation at 95°C for 10 s, annealing at 58°C for 10 s and elongation at 72°C for 4 s. The LightCycler 3.5 program provided by the manufacturer carried out the melting curve. The samples with similar Tm to the standard PK-9 methylation cDNA were judged as being positive for aberrant methylation of *TFPI-2*. For the internal reference gene *MYOD1*, the primers were designed to amplify and detect a region of the gene that is devoid of CpG nucleotides. Thus, amplification of *MYOD1* by quantitative real-time MSP occurs independently of its methylation status, whereas the amplification of *TFPI-2* is proportional to the degree of cytosine methylation within the *TFPI-2* promoter. The methylation ratio was defined as the ratio of the fluorescence signal for the real-time Q-MSP of *TFPI-2* generated by SYBR Green I, to those of the converted *MYOD1* templates that were fully methylated at the primer site.[33] Real-time Q-MSP primers for *MYOD1* were designed as methylation forward, 5′-CCAACTCCAAATCCCCTCTCTAT-3′, and reverse, 5′-TGATTAATTTAGATTGGGTTTAGAGAAGGA-3′ (gene bank: AF027148), in accordance with a previous report.[34]

Statistical methods. Experimental results were expressed as the mean ± SEM. The difference between the means was evaluated with the Mann–Whitney *U*-test. $P < 0.05$ was considered to be statistically significant. The statistical analysis was carried out using StatView-5.0 (SAS Institute, Tokyo, Japan).

Results

Expression of TFPI-2 mRNA and aberrant methylation of *TFPI-2* in the pancreatic cancer cell lines by quantitative analysis. Measurement of TFPI-2 mRNA was carried out by quantitative RT-PCR. Serial 10-fold dilutions of BxPC-3 cDNA were subjected to real-time RT-PCR to construct standard curves (Fig. 1a). A positive result was defined as one with the same Tm as for *TFPI-2* cDNA. The Tm of RT-PCR products for *TFPI-2* was 83.54°C (data not shown). TFPI-2 mRNA was expressed in PK-8, KLM-1, Mia-PaCa-2 and BxPC-3 cell lines (Fig. 1c). 18S was also measured by real-time RT-PCR as an internal control (data not shown). As shown in Fig. 1b, using a *TFPI-2* methylation-specific primer, the aberrant methylation of *TFPI-2* was investigated by real-time MSP. Serial 10-fold dilutions of PK-9 methylation cDNA were subjected to real-time MSP with the samples and gave standard curves. The positive results of samples were assessed according to the Tm of the methylated *TFPI-2* cDNAs. The Tm of hypermethylated *TFPI-2* was 82.30°C (data not shown). Expression of aberrantly methylated *TFPI-2* was recognized in all cell lines, except for PK-8 and PANC-1. *MYOD1* acted as an internal control using the same DNA modified by bisulfite. As shown in Fig. 1c, the ratios of TFPI-2:18S mRNA and hypermethylated TFPI-2:MYOD1 were calculated. There were four patterns of TFPI-2 expression and aberrant methylation in the PCa cell lines examined. The first pattern was TFPI-2 mRNA expression with no aberrant methylation (PK-8). The second was low expression of TFPI-2 mRNA and aberrant methylation of *TFPI-2* (KLM-1, Mia-PaCa-2 and BxPC-3). The third was expression of aberrantly methylated *TFPI-2*, with no TFPI-2 mRNA expression (PK-45H, PK-59, PK-1 and PK-9). The fourth was neither TFPI-2 mRNA expression nor aberrant methylation of *TFPI-2* expression (PANC-1).

Aberrant methylation of *TFPI-2* in PPJ of various pancreatic diseases and primary pancreatic cancer tissues by MSP analysis. As shown in Fig. 2a, there was aberrant methylation of *TFPI-2* in the PPJ obtained endoscopically from the patients with PCa, IPMN and CP, and primary PCa tissues by MSP using two pairs of specific PCR primers. The incidences of aberrant methylation for *TFPI-2* in the PPJ from the patients with PCa, IPMN and CP were 58.3% (21/36), 17.6% (3/17) and 4.8% (1/21), respectively (Fig. 2b). The incidence of aberrant methylation of *TFPI-2* in PCa was more frequent than that with IPMN or CP. Moreover, among IPMN, the incidence of hypermethylation of *TFPI-2* was 14.3% (1/7) in benign IPMN and 20% (2/10) in malignant IPMN. According to statistical analysis, the hypermethylation rate of *TFPI-2* in PPJ with PCa was significantly higher than that with IPMN or CP, respectively ($P < 0.01$, $P < 0.001$). However, there was no significant difference between IPMN and CP. In addition, there was no significant difference between benign IPMN and malignant IPMN.

Among 36 patients with PCa, 20 patients were confirmed as PCa histologically. Twelve of the 20 patients with PCa had positive results for aberrant methylation of *TFPI-2* in the PPJ. Aberrant methylation of *TFPI-2* was recognized in all of the four available PCa tissue samples examined out of the 12 PCa patients, as shown in Fig. 2a. Therefore, aberrant methylation of *TFPI-2* was confirmed in the primary PCa tissues examined in line with the positive results of PPJ.

Aberrant methylation of *TFPI-2* in PPJ of various pancreatic diseases by Q-MSP analysis. Aberrant methylation of *TFPI-2* was measured in the PPJ obtained endoscopically from patients with PCa, IPMN and CP by Q-MSP. The Tm of hypermethylated

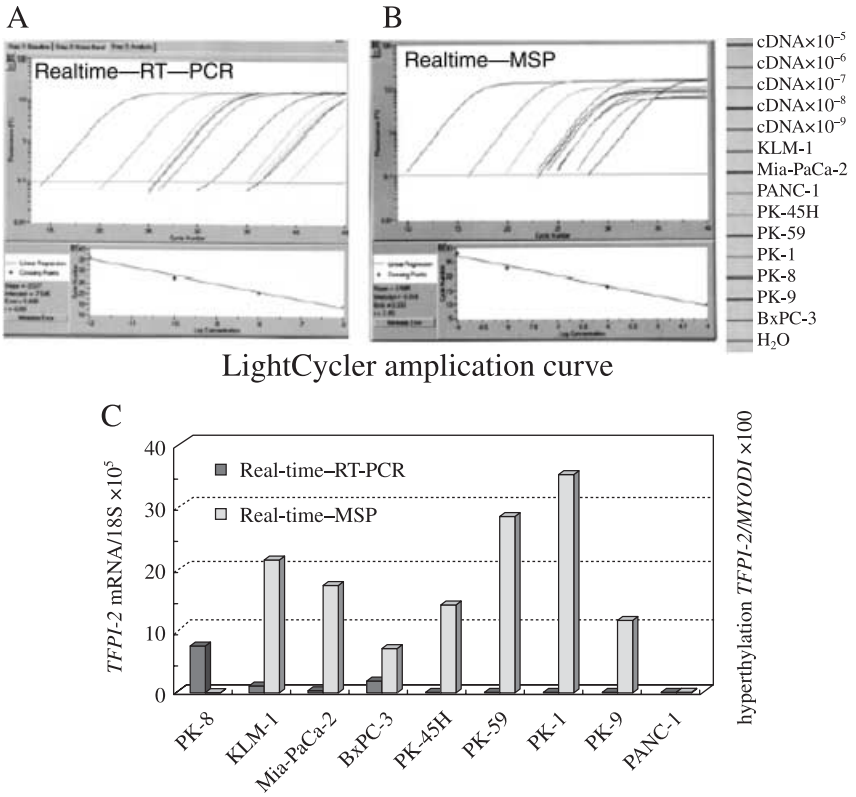

Fig. 1. The amplification curves of tissue factor pathway inhibitor 2 (TFPI-2) mRNA expression and *TFPI-2* aberrant methylation in the pancreatic cancer cell lines by quantitative analysis. (a) Serial 10-fold dilutions of the BxPC-3 cDNA gave the standard curves. The positive results of samples were judged according to the same melting temperatures and similar melting curves as the *TFPI-2* cDNAs. TFPI-2 mRNA was expressed in KLM-1, Mia-PaCa-2, PK-8 and BxPC-3 cell lines. (b) Serial 10-fold dilutions of the PK-9 methylation cDNA gave the standard curves. The positive results of samples were judged according to the same melting temperature and a similar melting curve as the methylated *TFPI-2* cDNAs. The aberrant methylation of *TFPI-2* was detected in KLM-1, Mia-PaCa-2, PK-45H, PK-59, PK-1, PK-9 and BxPC-3 cell lines. (c) The ratios of TFPI-2:18S mRNA and hypermethylated *TFPI-2*:*MYOD1* in pancreatic cancer cell lines were compared. The expression of TFPI-2 mRNA showed an inverse correlation to the aberrant methylation of *TFPI-2* by real-time analysis.

TFPI-2 was 82.30°C (data not shown). The ratios of hypermethylated *TFPI-2*:*MYOD1* in the PPJ of PCa, IPMN and CP were calculated (Fig. 3a). The ranges of the data for PCa, IPMN and CP were from 0.005 to 27.84, from 0 to 4.5 and from 0 to 6.82, respectively. The mean ratios of hypermethylated *TFPI-2*:*MYOD1* in the PPJ of PCa, IPMN and CP were 6.62 ± 7.20, 0.58 ± 1.02 and 1.28 ± 1.73, respectively. However, the mean ratio of aberrantly methylated *TFPI-2*:*MYOD1* in the PPJ was 0.136 ± 0.07 in benign IPMN and 0.884 ± 0.42 in malignant IPMN. In addition, it was 4.827 ± 2.191 for the PCa patients with liver metastasis. The hypermethylation ratio of *TFPI-2*:*MYOD1* in PCa was significantly higher than that in IPMN ($P < 0.01$) or CP ($P < 0.05$). There was no significant difference between IPMN and CP. There was also no significant difference between benign IPMN and malignant IPMN.

To improve the diagnostic accuracy, in accordance with the receiver operating characteristic curve analysis method, 2.5 was selected to be a suitable cut-off value in this study (Fig. 3b). Using this cut-off value, the aberrant methylation of *TFPI-2* in PCa, IPMN and CP was 62.1% (18/29), 5.9% (1/17) and 14.3% (3/21), respectively (Fig. 3c). The incidence of *TFPI-2* methylation was 0% (0/7) in benign IPMN and 10% (1/10) in malignant IPMN. The incidence of hypermethylated *TFPI-2* in the PPJ of PCa was significantly higher than that of IPMN ($P < 0.001$) or CP ($P < 0.001$). There was no significant difference between IPMN and CP. In addition, there was no significant difference between benign IPMN and malignant IPMN.

Correlation of the aberrant methylation of *TFPI-2* in PPJ with the clinicopathological parameters of pancreatic carcinoma between MSP and Q-MSP. We evaluated the aberrant methylation of *TFPI-2* in the PPJ aspirated endoscopically by MSP and Q-MSP and compared the results with the clinicopathological parameters of PCa (Table 2). The incidence of *TFPI-2* hypermethylation was 83.3% (5/6) in stages I + II + III and 53.3% (16/30) in stages

Table 2. Comparison of clinicopathological factors with pancreatic carcinoma patients for aberrant methylation of tissue factor pathway inhibitor 2 (*TFPI-2*) in pure pancreatic juice

Clinicopathological factor	MSP positive		Q-MSP positive	
	n	%	*n*	%
Clinical stage				
I + II + III	5/6	83.3	3/6	50.0
IVa + IVb	16/30	53.3	26/30	86.7
TS				
1 +2 + 3	19/33	57.6	27/33	81.8
4	2/3	66.7	2/3	66.7
Location				
H	11/16	68.8	14/16	87.5
B	10/16	62.5	13/16	87.5
T	0/4	0	2/4	50.0
Histological type				
G1	3/4	75.0	4/4	100
G2	6/11	54.5	6/11	54.5
G3	2/6	33.3	6/6	100
Live metastasis age (years)†				
<50	2/3	66.7	3/3	100
50 ≤ 60	7/12	58.3	8/12	66.7
60 ≤ 70	4/8	50	8/8	100
≥70	8/13	61.5	10/13	76.9

†Quantitative MSP (Q-MSP) for liver metastasis versus methylation-specific polymerase chain reaction (MSP) for liver metastasis ($P < 0.05$). No *P*-values were significant. B, body; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; H, head; T, tail; TS, tumor size.

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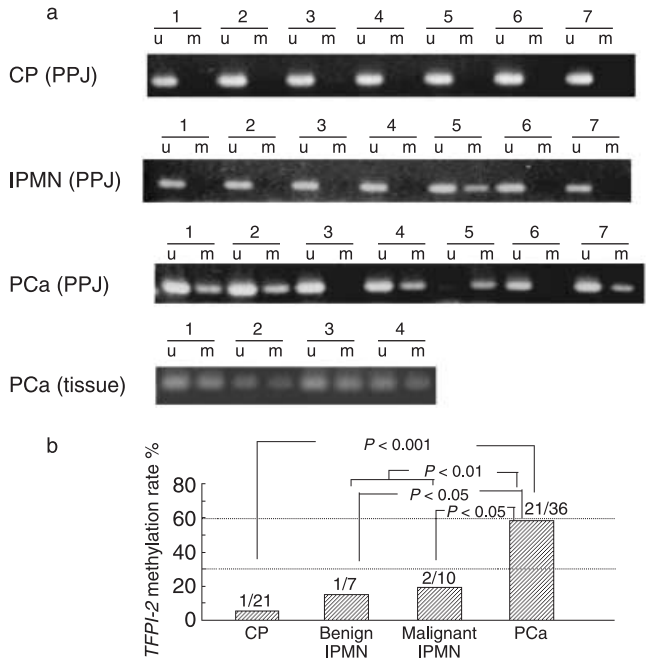

Fig. 2. Detection of the aberrant methylation of tissue factor pathway inhibitor 2 (*TFPI-2*) in pure pancreatic juice (PPJ) from the patients with pancreatic adenocarcinoma (PCa), intraductal papillary mucinous neoplasm (IPMN) and chronic pancreatitis (CP), and primary PCa tissues by methylation-specific polymerase chain reaction (MSP) analysis. (a) Using methylation and unmethylation specific primers, the 95-bp methylated PCR product and 89-bp unmethylated PCR product were detected in the PPJ from patients with PCa, IPMN and CP, and primary PCa tissues. (b) The aberrant methylation rate of *TFPI-2* in PCa, IPMN and CP was 58.3% (21/36), 17.6% (3/17) and 4.8% (1/21), respectively. The hypermethylation rate of *TFPI-2* was 14.3% (1/7) in benign IPMN and 20% (2/10) in malignant IPMN. The hypermethylation rate of *TFPI-2* in PCa was significantly higher than that in IPMN or CP, respectively ($P < 0.01$, $P < 0.001$).

IVa + IVb by MSP, whereas it was 50% (3/6) in stages I + II + III and 86.7% (26/30) in stages IVa + IVb by Q-MSP. There was no significant difference between stages I + II + III and stages IVa + IVb. However, the aberrant methylation of *TFPI-2* in the PPJ from the PCa patients with liver metastasis was extremely high (up to 100% [6/6]) by Q-MSP. The sensitivity, specificity and efficiency of liver metastasis with *TFPI-2* methylation were 33.3%, 35.7% and 35.3% by MSP, whereas they were 100%, 27.6% and 40.0% by Q-MSP. Q-MSP was better than MSP for detecting liver metastasis ($P < 0.05$). In contrast, there was no significant difference between *TFPI-2* hypermethylation by MSP and Q-MSP assay, and tumor size, tumor location, histological type or age, respectively.

Discussion

We have shown aberrant methylation in the promoter region of *TFPI-2* in various pancreatic diseases and PCa cell lines. A relative decrease of TFPI-2 mRNA synthesis was demonstrated in this study in 44.4% of PCa cell lines using a real-time RT-PCR assay. Real-time RT-PCR is a sensitive and reproducible method, compared with Northern blot analysis, semiquantitative RT-PCR and competitive RT-PCR. There were four patterns for the expression of TFPI-2 mRNA and hypermethylated *TFPI-2* in the PCa cell lines examined. The expression of TFPI-2 mRNA was inversely proportional to that of aberrantly methylated *TFPI-2* in most of the PCa cell lines examined. These results suggest that hypermethylation of *TFPI-2* is involved in silencing TFPI-2

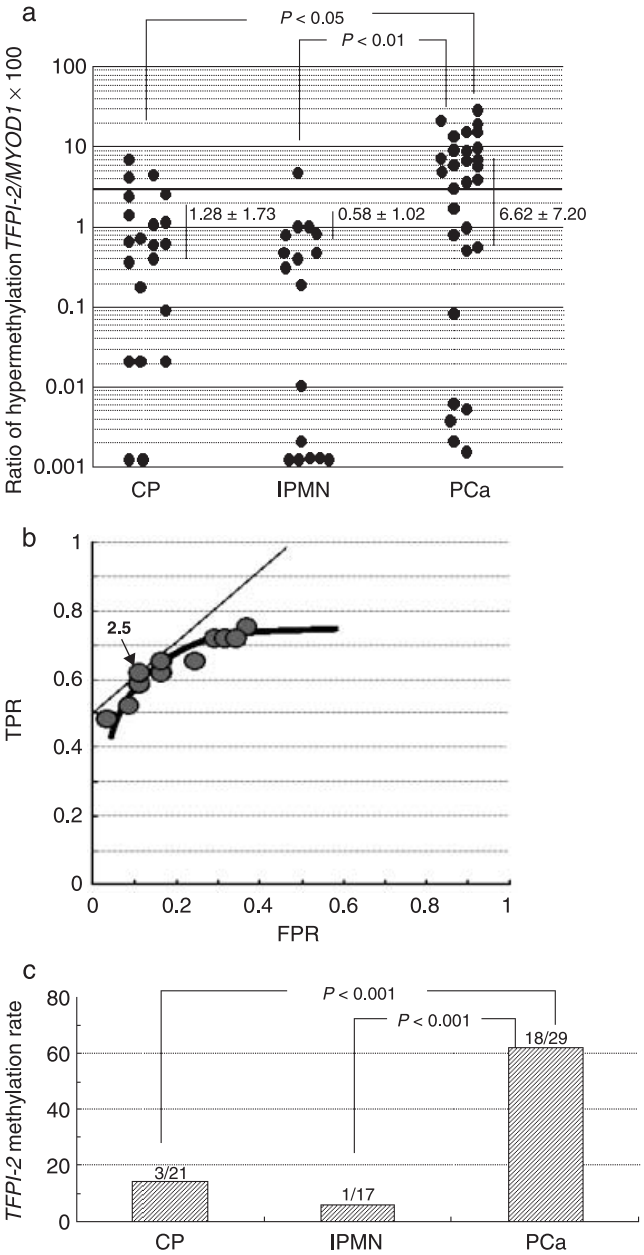

Fig. 3. Aberrant methylation of tissue factor pathway inhibitor 2 (*TFPI-2*) in pure pancreatic juice (PPJ) from patients with pancreatic adenocarcinoma (PCa), intraductal papillary mucinous neoplasm (IPMN) and chronic pancreatitis (CP) by real-time methylation-specific polymerase chain reaction (MSP) analysis. (a) The ratio of hypermethylated *TFPI-2*:*MYOD1* in PCa, IPMN and CP was 6.62 ± 7.20, 0.58 ± 1.02 and 1.28 ± 1.73, respectively. The hypermethylation in PCa was significantly higher than that in IPMN ($P < 0.01$) or CP ($P < 0.05$). There was no significant difference between IPMN and CP. (b) The receiver operating characteristic curve of *TFPI-2* methylation in PCa diagnosis from the other pancreatic diseases. The suitable cut-off value was selected as 2.5. FPR, false positive rate; TPR, true positive rate. (c) Using 2.5 as a suitable cut-off value, the aberrant methylation rates of *TFPI-2* in PCa, IPMN and CP was 62.1% (18/29), 5.9% (1/17) and 14.3% (3/21), respectively. The incidence of hypermethylation for *TFPI-2* in PCa was significantly higher than that in IPMN ($P < 0.001$) or CP ($P < 0.001$).

mRNA in the PCa cell lines. However, neither TFPI-2 mRNA nor hypermethylated *TFPI-2* was detected in the PANC-1 cell line. These findings suggest that besides methylation, there were other mechanisms involved in silencing TFPI-2 mRNA in the

PANC-1 cell line. Besides aberrant methylation, there are many mechanisms involved in silencing tumor genes, such as deletion, mutation, histone deacetylation and phosphorylation. It is known that the mechanisms of *TFPI-2* silencing are related to hypermethylation, histone deacetylation and the Ras/Raf/MEK/ERK pathway.[27,35–37]

Characterization of the tumor-related genes silenced or activated by aberrant methylation and their roles in human tumorigenesis may afford significant insight into the peculiar biology associated with PCa. The aberrant methylation of *TFPI-2* was detected in PPJ from patients with the PCa, IPMN and CP. The hypermethylation of *TFPI-2* in PCa was significantly higher than that in IPMN or CP according to both MSP and Q-MSP assays. These results suggest that the aberrant methylation of *TFPI-2* is actually involved in PCa development and progression, and is related to the silencing of TFPI-2 mRNA expression. Because the MSP results for *TFPI-2* hypermethylation in PCa were significantly higher than those in IPMN and CP, the MSP assay can be useful in the diagnosis of PCa. The aberrant methylation of *TFPI-2* in PCa by real-time MSP assay was not significantly higher than that by MSP analysis.

Tissue factor pathway inhibitor 2 suppresses the degradation of ECM and reduces tumor invasion as a serine proteinase inhibitor. Silencing *TFPI-2* causes the loss of its original functions. Rollin *et al*. reported that decreased *TFPI-2* gene expression and *TFPI-2* hypermethylation are more frequently associated with advanced stages of non-small cell lung cancer, and that the *TFPI-2* gene promoter is more frequently hypermethylated in patients with lymph node metastases.[38] However, Konduri *et al*. showed that TFPI-2 mRNA is more highly expressed in low-grade gliomas than in high-grade gliomas.[35,37] We showed that the aberrant methylation rate of *TFPI-2* in the PPJ was 100% (6/6), as observed in PCa patients with liver metastasis, and 86.7% (26/30) in stages IVa + IVb of PCa by Q-MSP assay. The hypermethylation of *TFPI-2* in the PPJ is thought to be one of the most sensitive markers in the diagnosis of liver metastasis from PCa. It may be used as a predictor of cancer progression and to speculate about prognosis using an endoscopically feasible approach.

There are many hypermethylated genes involved in the course of PCa development, such as *SARP2*, *ppENK*, *cyclin D2*, *NTPX2* and *CLDN5*.[11,39–41] The incidence of aberrant promoter methylation of *Cyclin D2* in PCa and IPMN was 65.1% (71/109) and 50% (23/46), respectively.[40] The hypermethylation of *SARP2*, *NTPX2* and *CLDN5* in PCa, IPMN and CP was 75%, 85% and 5%, 71%, 53% and 32%, and 26%, 47% and 16%, respectively (unpublished data). Compared with these genes, the aberrant methylation of *TFPI-2* is more specific to PCa. Sato *et al*. reported that the hypermethylated *TFPI-2* was detected in 57% of PPJ obtained at operation of PCa patients, and in 0% of benign pancreatic disorder patients.[39] Therefore, the detection of aberrantly methylated *TFPI-2* in PPJ may be used in the diagnosis of PCa, especially in the differential diagnosis of benign and malignant pancreatic diseases.

Intraductal papillary mucinous neoplasm showed a broad spectrum of cytological and architectural atypia, ranging from benign (hyperplasia and adenoma) to malignant (carcinoma), and may be considered as a precancerous lesion. In the present study, IPMN was divided into benign and malignant groups under diagnostic criteria. Although our data showed that the aberrant methylation rate of *TFPI-2* in malignant IPMN was slightly higher than that in benign IPMN, there was no significant difference between them (Fig. 2b). In contrast, Sato *et al*.[29] divided IPMN into two groups according to histological grade: those classified as adenoma and borderline (low-grade IPMN) and those classified as *in situ* carcinoma (high-grade IPMN). This classification of IPMN was not different from ours. High-grade IPMN were nearly IPMN-derived carcinoma. They reported that aberrant methylation of *TFPI-2* was detected at a significantly higher frequency in high-grade IPMN than in low-grade IPMN (85% vs 17%: $P = 0.0002$) by MSP. In our study, low incidence of *TFPI-2* methylation in the PPJ from both benign and malignant IPMN groups by both MSP and Q-MSP suggests that the cause of the low incidence of *TFPI-2* methylation was that no IPMC manifested histologically in the malignant IPMN group. Moreover, the aberrant methylation rate of *TFPI-2* in the PPJ was 100% (6/6) in the PCa patients with liver metastasis, and 86.7% (26/30) in stage IVa + IVb of PCa by Q-MSP. These results indicate that aberrant methylation of *TFPI-2* may be specific to PCa and later events in the progression of PCa.

In the present study, we compared the clinical usefulness of aberrant methylation of *TFPI-2* in the PPJ for the diagnosis of PCa for between MSP and Q-MSP. Using the *MYOD1* gene as an internal reference, 2.5 was selected as a suitable cut-off value for the ratio of *TFPI-2*:*MYOD1* under receiver operator characteristic curve analysis with Q-MSP. The incidence of *TFPI-2* hypermethylation was 83.3% in stages I–III and 53.3% in stage IV by MSP, whereas it was 50% in stages I–III and 86.7% in stage IV by Q-MSP. There was no statistical significance between MSP and Q-MSP. These results suggest that Q-MSP was not always superior to MSP for clinical utility in the diagnosis and progression of PCa. In addition, the MSP method for the aberrant methylation of TFPI-2 in the PPJ was simple and convenient and useful for the diagnosis of PCa.

In conclusion, we have shown that hypermethylation of *TFPI-2* in the PPJ was detected in 58.3% (21/36) of PCa, 17.6% (3/17) of IPMN and 4.8% (1/21) of CP by MSP assay. The hypermethylation of *TFPI-2* was higher in liver metastasis patients according to Q-MSP assay. Detection of the aberrant methylation of *TFPI-2* in PPJ may be useful in the diagnosis of PCa, especially in the differential diagnosis of benign and malignant pancreatic diseases, and in the progression of PCa using an endoscopically feasible approach.

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References

1 Bramhall SR, Allum WH, Jones AG, Allwood A, Cummins C, Neoptolemos JP. Treatment and survival in 13 560 patients with pancreatic cancer, and incidence of the disease, in the West Midlands: an epidemiological study. *Br J Surg* 1995; **82**: 111–15.
2 Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. *CA Cancer J Clin* 2003; **53**: 5–26.
3 Fernandez E, La Vecchia C, Porta M, Negri E, Lucchini F, Levi F. Trends in pancreatic cancer mortality in Europe, 1955–89. *Int J Cancer* 1994; **57**: 786–92.
4 Parkin DM, Bray FI, Devesa SS. Cancer burden in the year 2000: the global picture. *Eur J Cancer* 2001; **37** (Suppl. 8): S4–66.
5 Neoptolemos JP, Stocken DD, Friess H *et al*. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med* 2004; **350**: 1200–10.
6 Sawabu N, Watanabe H, Yamaguchi Y, Ohtsubo K. Serum tumor markers and molecular biological diagnosis in pancreatic cancer. *Pancreas* 2004; **28**: 263–7.
7 Ohtsubo K, Watanabe H, Yamaguchi Y *et al*. Abnormalities of tumor suppressor gene *p16* in pancreatic carcinoma: immunohistochemical and genetic findings compared with clinicopathological parameters. *J Gastroenterol* 2003; **38**: 663–71.
8 Ha A, Watanabe H, Yamaguchi Y *et al*. Usefulness of supernatant of pancreatic juice for genetic analysis of K-*ras* in diagnosis of pancreatic carcinoma. *Pancreas* 2001; **23**: 356–63.

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9 Yan L, McFaul C, Howes N *et al*. Molecular analysis to detect pancreatic ductal adenocarcinoma in high-risk groups. *Gastroenterology* 2005; **128**: 2124–30.
10 Sato N, Ueki T, Fukushima N *et al*. Aberrant methylation of CpG islands in intraductal papillary mucinous neoplasms of the pancreas. *Gastroenterology* 2002; **123**: 365–72.
11 Sato N, Fukushima N, Maitra A *et al*. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. *Cancer Res* 2003; **63**: 3735–42.
12 Watanabe H, Sawabu N, Ohta H *et al*. Identification of K-*ras* oncogene mutations in the pure pancreatic juice of patients with ductal pancreatic cancers. *Jpn J Cancer Res* 1993; **84**: 961–5.
13 Watanabe H, Sawabu N, Songür Y *et al*. Detection of K-*ras* point mutations at codon 12 in pure pancreatic juice for the diagnosis of pancreatic cancer by PCR-RFLP analysis. *Pancreas* 1996; **12**: 18–24.
14 Watanabe H, Miyagi C, Yamaguchi Y *et al*. Detection of K-*ras* point mutations at codon 12 in pancreatic juice for the diagnosis of pancreatic cancer by hybridization protection assay: a simple method for the determination of the types of point mutations. *Jpn J Cancer Res* 1996; **87**: 466–74.
15 Watanabe H, Yamaguchi Y, Ha A *et al*. Quantitative determination of K-*ras* mutations in pancreatic juice for diagnosis of pancreatic cancer using hybridization protection assay. *Pancreas* 1998; **17**: 341–7.
16 Watanabe H, Ha A, Hu YX *et al*. K-*ras* mutations in duodenal aspirate without secretin stimulation for screening of pancreatic and biliary tract carcinoma. *Cancer* 1999; **86**: 1441–8.
17 Okai T, Watanabe H, Yamaguchi Y *et al*. EUS and K-*ras* analysis of pure pancreatic juice collected via a duodenoscope after secretin stimulation for diagnosis of pancreatic mass lesion: a prospective study. *Gastrointest Endosc* 1999; **50**: 797–803.
18 Yamaguchi Y, Watanabe H, Songür Y *et al*. Detection of mutations of *p53* tumor suppressor gene in pancreatic juice and its application to diagnosis of patients with pancreatic cancer: comparison with K-*ras* mutation. *Clin Cancer Res* 1999; **5**: 1147–53.
19 Ha A, Watanabe H, Yamaguchi Y *et al*. Usefulness of supernatant of pancreatic juice for genetic analysis of K-*ras* in diagnosis of pancreatic carcinoma. *Pancreas* 2001; **23**: 256–63.
20 Wang Y, Yamaguchi Y, Watanabe H, Ohtsubo K, Wakabayashi T, Sawabu N. Usefulness of *p53* gene mutations in the supernatant of bile duct for diagnosis of biliary tract carcinoma: comparison with K-*ras* mutation. *J Gastroenterol* 2002; **37**: 831–9.
21 Wang Y, Yamaguchi Y, Watanabe H, Ohtsubo K, Motoo Y, Sawabu N. Detection of *p53* gene mutations in the supernatant of pancreatic juice and plasma from patients with pancreatic carcinomas. *Pancreas* 2004; **28**: 13–19.
22 Kong D, Ma D, Bai H *et al*. Expression and characterization of the first kunitz domain of human tissue factor pathway inhibitor-2. *Biochem Biophys Res Commun* 2004; **324**: 1179–85.
23 Konduri SD, Tasiou A, Chandrasekar N, Rao JS. Overexpression of tissue factor pathway inhibitor-2 (TFPI-2), decreases the invasiveness of prostate cancer cells *in vitro*. *Int J Oncol* 2001; **18**: 127–31.
24 Tanaka Y, Utsumi J, Matsui M *et al*. Purification, molecular cloning, and expression of a novel growth-promoting factor for retinal pigment epithelial cells, REF-1/TFPI-2. *Invest Ophthalmol Vis Sci* 2004; **45**: 245–52.
25 Yanamandra N, Kondraganti S, Gondi CS *et al*. Recombinant adeno-associated virus (rAAV) expressing TFPI-2 inhibits invasion, angiogenesis and tumor growth in a human glioblastoma cell line. *Int J Cancer* 2005; **115**: 998–1005.
26 Rao CN, Mohannam S, Puppala A, Rao JS. Regulations of ProMMP-1 and ProMMP-3 activation by tissue factor pathway inhibitor-2/matrix-associated serine protease inhibitor. *Biochem Biophys Res Commun* 1999; **255**: 94–8.
27 Steiner FA, Hong JA, Fischette MR *et al*. Sequential 5-Aza 2′-deoxycytidine/depsipeptide FK228 treatment induces tissue factor pathway inhibitor 2 (TFPI-2) expression in cancer cells. *Oncogene* 2005; **24**: 2386–97.
28 Hube F, Reverdiau P, Iochmann S, Rollin J, Cherpi-Antar C, Gruel Y. Transcriptional silencing of the *TFPI-2* gene by promoter hypermethylation in choriocarcinoma cells. *Biol Chem* 2003; **384**: 1029–34.
29 Sato N, Parker AR, Fukushima N *et al*. Epigenetic inactivation of *TFPI-2* as a common mechanism associated with growth and invasion of pancreatic ductal adenocarcinoma. *Oncogene* 2005; **24**: 850–8.
30 Hermanek P, Sobin LH. *TNM Classification of Malignant Tumors*, 4th edn. Place?: Springer Verlag, 1987.
31 Solcia E, Capella C, Klöppel G. Tumors of the pancreas. In: *Atlas of Tumor Pathology, 3rd Series, Fascicte 20*. Washington, DC: Armed Forces Institute of Pathology, 1997; 103–14.
32 Honma T, Harada H, Koizumi M. Diagnostic criteria for chronic pancreatitis by the Japan Pancreas Society. *Pancreas* 1997; **15**: 14–15.
33 Yegnasubramanian S, Kowalski J, Gonzalgo ML *et al*. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res* 2004; **64**: 1975–86.
34 Bastian PJ, Ellinger J, Wellmann A *et al*. Diagnostic and prognostic information in prostate cancer with the help of a small set of hypermethylated gene loci. *Clin Cancer Res* 2005; **11**: 4097–106.
35 Konduri SD, Srivenugopal KS, Yanamandra N *et al*. Promoter methylation and silencing of the tissue factor pathway inhibitor-2 (*TFPI-2*), a gene encoding an inhibitor of matrix metalloproteinases in human glioma cells. *Oncogene* 2003; **22**: 4509–16.
36 Rao CN, Segawa T, Navari JR *et al*. Methylation of *TFPI-2* gene is not the sole cause of its silencing. *Int J Oncol* 2003; **22**: 843–8.
37 Konduri SD, Yanamandra N, Dinh DH *et al*. Physiological and chemical inducers of tissue factor pathway inhibitor-2 in human glioma cells. *Int J Oncol* 2003; **22**: 1277–83.
38 Rollin J, Iochmann S, Blechet C *et al*. Expression and methylation status of tissue factor pathway inhibitor-2 gene in non-small-cell lung cancer. *Br J Cancer* 2005; **92**: 775–83.
39 Sato N, Maitra A, Fukushima N *et al*. Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. *Cancer Res* 2003; **63**: 4158–66.
40 Matsubayashi H, Sato N, Fukushima N *et al*. Methylation of *cyclin D2* is observed frequently in pancreatic cancer but is also an age-related phenomenon in gastrointestinal tissues. *Clin Cancer Res* 2003; **9**: 1446–52.
41 Ueki T, Toyota M, Sohn T *et al*. Hypermethylation of multiple genes in pancreatic adenocarcinoma. *Cancer Res* 2000; **60**: 1835–9.