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Adenovirus-mediated gene transfer of tissue factor pathway inhibitor-2 inhibits gallbladder carcinoma growth *in vitro* and *in vivo*

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Tissue factor pathway inhibitor-2 (TFPI-2) has been identified as a tumor suppressor gene in several types of cancers, but its role in gallbladder carcinoma (GBC) is yet to be determined. In the present study, TFPI-2 expression in GBC tissues was examined, and its inhibitory activities against GBC growth were evaluated *in vitro* and *in vivo* after adenovirus-mediated gene transfer of TFPI-2 (Ad5-TFPI-2) was constructed to restore the expression of TFPI-2 in GBC cell lines (GBC-SD, SGC-996, NOZ) and xenograft tumors. Immunohistochemical staining showed that TFPI-2 was significantly downregulated in GBC tissue specimens. Ad5-TFPI-2 could significantly inhibit GBC growth both *in vitro* and *in vivo*. Apoptosis analysis and western blotting assay demonstrated that Ad5-TFPI-2 could induce the apoptosis of both GBC cell lines and tissues by promoting the activities of cytochrome *c*, Bax, caspase-3 and -9 and suppressing Bcl-2 activity. These data indicated that TFPI-2 acts as a tumor suppressor in GBC, and may have a potential role in gene therapy for GBC. (*Cancer Sci* 2012; 103: 723–730)

Gallbladder carcinoma (GBC) is a relatively uncommon but highly fatal cancer. Due to its late diagnosis, rapid progression, poor prognosis and high recurrence,[1] GBC has always been posing challenges to clinicians. Until now, radical resection remains the only potentially curative therapy for GBC. Masked by the non-specific symptoms, most GBC cases are diagnosed in their late stage, and for these advanced GBC, radical or extended resection can only be performed in <30% cases. There is no guarantee that long-term survival can be prolonged or that recurrences can be avoided even after surgery.[2] In addition, radiotherapy and traditional chemotherapy for GBC are also far from effective.[3,4] The next therapeutic breakthrough is largely dependent on how we better understand the etiology, pathogenesis and development for GBC.[5,6]

It has been demonstrated that inactivation of tumor suppressor genes including p53, p16 and p21[7–9] contribute to GBC tumorigenesis. Gene therapy may offer new strategies to treat GBC. Herein, we focused on a novel tumor suppressor gene, tissue factor pathway inhibitor-2 (TFPI-2), which is a member of Kunitz-type serine protease inhibitor family.[10] TFPI-2 is usually produced by endothelial cells (ECs) and secreted into ECM to fulfill its functions. It can inhibit a wide variety of serine proteinases including trypsin, plasmin, chymotrypsin, and factor XIa[11] as well as MMPs.[12] Recently, the role of TFPI-2 has been investigated in several different cancers. TFPI-2 expression was found to be abundant in many normal tissues, while its expression dramatically decreased in corresponding cancer tissues.[13] Restoration of TFPI-2 expression in tumors could inhibit tumor growth and progression by suppressing the activities of plasmin, MMPs and vascular endothelial growth factor (VEGF).[14] However, most studies have paid great attention to its extracellular role in cancer biology. In the present study, its intracellular role in GBC was first investigated, including the expression of TFPI-2 in GBC tissues and cell lines and its functions against tumor growth *in vitro* and *in vivo* by virtue of restoring TFPI-2 expression in GBC cell lines and xenograft tumors via adenovirus-mediated gene transfer of TFPI-2 (Ad5-TFPI-2). The part of TFPI-2 played in apoptosis was also investigated in the GBC-SD cell line.

Materials and Methods

Patients and tissue specimens. The study was approved by the ethics committee of Xinhua hospital and all patients provided informed consent. Cancer tissue specimens were obtained from 34 cases of GBC patients who underwent radical cholecystectomy (without prior radiotherapy or chemotherapy) between 2008 and 2010 at the Department of General Surgery, Xinhua hospital, School of Medicine, Shanghai Jiaotong University, China. According to the Union for International Cancer Control (UICC) staging system, six (17%), eight (24%), 12 (35%), eight (24%) cases were diagnosed with stages I, II, III and IV disease, respectively, and 16 cases were diagnosed with lymph node metastasis. In addition, 24 patients with gallbladder polyp (GBP) who underwent simple cholecystectomy were included as controls and the pathologic types involved cholesterol polyp, adenomatous hyperplasia, and gallbladder adenomyomatosis. All diagnoses of GBC, GBP and lymph node metastasis were confirmed by histopathological examination and all tissue samples were fixed in 4% formalin immediately after removal and embedded with paraffin for immunohistochemical staining.

Construction of recombinant adenovirus. Construction of the recombinant adenoviral serotype 5 vector carrying either human TFPI-2 cDNA (Ad5-TFPI-2) or GFP gene (Ad5-GFP) were performed by the Laboratory of Gene and Viral Therapy, Eastern Hepatobiliary Surgical Hospital, the Second Military Medical University. Mouse cytomegalovirus (mCMV) was used as the promoter to drive the synthesis of TFPI-2. In brief, the TFPI-2 cDNA was cloned into the plasmid containing the mCMV promoter (pSG-CMV) to produce pSG-TFPI-2 which was further co-transfected with the recombinant shuttle vector into human embryonic kidney 293 cells (HEK293) to produce Ad5-TFPI-2. The virus was identified correctly by restriction endonuclease digestion and RT-PCR, and in turn purified, amplified and titrated (2.0×10^{10} pfu/mL). Ad5-GFP expressing GFP protein was used as a control. All viruses were stored at −70°C until further use.

Cell culture and gene transduction. Three human GBC cell lines were used in this study. GBC-SD was purchased from Cell

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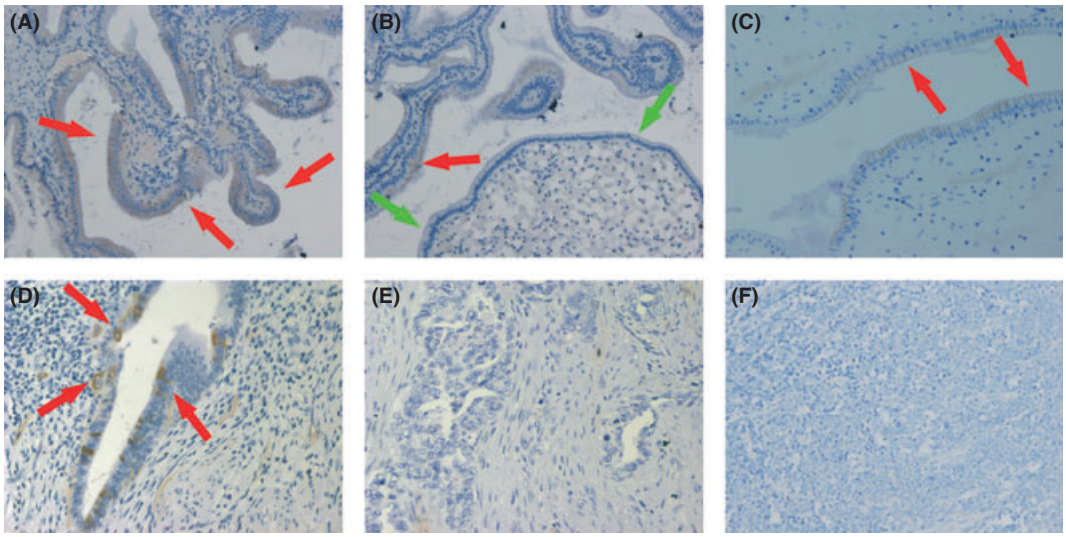

Fig. 1. Expression of tissue factor pathway inhibitor-2 (TFPI-2) in gallbladder carcinoma (GBC) tissues, lymph metastasis, normal tissues, and gallbladder polyp (GBP) tissues by immunohistochemical staining (×200). The percentage of positive-stained epithelial cells or cancer cells with >10% were considered as positive. Red arrow represents positive expression, and green arrow represents negative expression. (A) TFPI-2 expression in normal tissues. TFPI-2 staining was located in the cytoplasm of epithelial cells. (B) TFPI-2 expression was detected in epithelial cells of normal tissue but not in those of GBP tissues. (C) TFPI-2 expression was also detected in epithelial cells of GBP tissues. (D) TFPI-2 expression was detected in cancer cells of GBC tissues. (E) TFPI-2 expression was not detected in cancer cells of GBC tissues. (F) TFPI-2 expression was not detected in cancer cells of lymph metastasis.

Bank of the Chinese Academy of Sciences (Shanghai, China); SGC-996 was provided by Academy of Life Science, Tongji University (Shanghai, China); NOZ was purchased from the Health Science Research Resources Bank (Osaka, Japan). The cell lines were cultured in DMEM (Gibco BRL, Carlsbad, CA, USA) containing 15% FBS (HyClone, Logan, UT, USA), 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. For gene transduction, cells were grown to confluence and incubated with Ad5-TFPI-2 or Ad5-GFP, both of which were suitably diluted in serum-free medium at concentrations of desired MOI. After 12 h, the serum-free medium was replaced with fresh serum-containing medium and cells were incubated for desired time periods. Ad5-GFP and PBS were used as controls.

Animal experiment. Male athymic BALB/c nude mice (age: 5 weeks, weight: 20–25 g) were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Mice were housed under specific pathogen-free (SPF) conditions following the guidelines of the Ethics Committee of Xinhua Hospital, School of Medicine, Shanghai Jiaotong University. The xenograft tumor model was established by subcutaneous injection of GBC-SD cells *in vivo*. Cells were injected into the right flank of nude mice with a concentration of 1×10^7 cells/0.1 mL. 2×10^{10} pfu of Ad5-TFPI-2 or Ad5-GFP within 200 μL of PBS was intratumorally administered every 3 days when tumor volume reached 0.5 cm^3. All treatments were administered four times. The tumor volumes in each group were measured every 3 days after treatments using the following formula: volume = length × $width^2/2$. Three weeks after treatments, the mice were killed and the xenograft tumors were collected for further immunohistochemistry staining and TUNEL assay.

Immunohistochemical staining. The 4-μm-thick tissue sections were cut from paraffin-embedded xenograft tumors or human GBC tissue, deparaffinized within xylene, and rehydrated within a graded series of ethanol solutions. The endogenous peroxidase activity was halted by 3% (v/v) H_2O_2. In order to eliminate non-specific staining, those sections were incubated with 3% bovine plasma albumin at room temperature for 30 min first and then incubated with anti-human TFPI-2 monoclonal antibody (1:50 dilution) at 4°C overnight. After washing with TBS, the sections were incubated with secondary antibodies (1:100 dilution) for 15 min. Finally, they were incubated with peroxidase complex and visualized by 3,3′-diaminobenzidine tetrachloride (DAB).

RT-PCR. Total RNA was extracted from GBC-SD cells using Trizol reagent kit (Gibco BRL) according to the manufacturer's instructions. After quantification, complementary DNA (cDNA) was synthesized from 2 μg of total RNA using a Takara RNA PCR kit (Takara Bio Inc, Dalian, China) according to manufacturer's instructions. The primers used in study were designed by primer premier version 5.0 and synthesized by Sangon Biotech (Shanghai, China). The following sequences were selected: TFPI-2 forward: TCTATCCTCCAGCAAGCATCGTC; TFPI-2 reverse: AGGAGCCAACAGGAAATAACGC. The procedures were performed as follows: initial denaturation at 95°C for 3 min, 35 cycles of 30 s at 94°C, 30 s at 63°C for primer annealing, 30 s at 72°C for extension, followed by 5 min at 72°C for final extension. The PCR product was visualized by 1.5% agarose gel and stained with Ethidium Bromide. β-Actin was used as control.

Cell viability assay. The cell viability of three cell lines was determined by the water soluble tetrazolium (WST)-1 method

Table 1. Tissue factor pathway inhibitor-2 (TFPI-2) expression in normal tissues, gallbladder polyp (GBP) tissues and gallbladder carcinoma (GBC) tissues

TFPI-2 expression	Tissue specimens		
	Normal tissues	GBP tissues	GBC tissues
Positive rate % (*n*)	79.2% (19)	45.8% (11)	11.8% (4)

TFPI-2 expression was significantly decreased in GBC tissues when compared with GBP tissues and normal tissues ($P = 0.000$, chi-squared test).

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using WST-1 cell proliferation and cytotoxicity assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. In brief, 5×10^3 cells were seeded in 200 μL/well culture medium in 96-well plates for 24 h, and then treated with Ad5-TFPI-2 or Ad5-GFP, both of which were administered at various MOI (1, 10, 50, 100, 500). After 72 h, the cells were incubated with WST-1 reagent for 2 h at 37°C. The absorbance was examined at 450 nm with an automated microplate reader (Bio-Rad 5 Model 550, Bio-Rad, Hercules, CA, USA). The percent viability of cells was calculated using the following equation: cell viability = mean optical density (OD) of one experimental group/mean OD of the control group × 100%.

Apoptosis assay. The apoptosis of cell lines was examined by flow cytometry using APC-Annexin V/propidium iodide (PI) staining (BD Pharmingen, San Diego, CA, USA). Cells were cultured in six-well plates for 24 h and then incubated with Ad5-TFPI-2 or Ad5-GFP (MOI 50). After 72 h, at least 1×10^5 cells (including floating cells) were collected, centrifuged, washed twice with cold PBS, and resuspended in binding buffer. Double staining was performed with Annexin V/PI in a dark room at room temperature for 15 min, and all the samples were analyzed by flow cytometry within 1 h. Assessment of apoptosis in xenograft tumor tissues was performed by TUNEL method using FragEL DNA Fragmentation Detection Kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Counterstaining with methyl green was used to aid in the morphological evaluation and characterization of normal and apoptotic cells. Apoptotic cells were quantified by counting the number of TUNEL-stained nuclei, which was performed in randomly chosen fields under a light microscope. The data were analyzed with NIS-Elements BR3.0 software (Nikon, Kanagawa, Japan). The number of apoptotic cells per field was presented as mean ± SD. A dark brown DAB signal indicated positive staining while shades of blue to green signified a non-reactive cell. A positive control slide was also provided with the kit.

Western blotting. The expressions of TFPI-2, cytochrome *c*, p53, RB/pRB, Bax, Bcl-2, p21, p27, cleaved caspase-3 and cleaved caspase-9 in GBC-SD cells were examined by western blotting, and β-actin was used as a control. The mitochondria were isolated from the total cell lysates using cell mitochondria isolation kit (Beyotime) and the cytosol fraction was used to measure released cytochrome *c*. All the proteins were quantified using Bradford protein assay. Proteins were separated by 10–12% SDS-PAGE and transferred to PVDF transfer membranes (Millipore, Billerica, MA, USA). The blot was blocked with 5% non-fat dry milk and then incubated with corresponding primary antibodies (Santa Cruz Bio Inc, Santa Cruz, CA, USA) at 4°C overnight and with corresponding secondary antibodies (Santa Cruz Bio Inc) at room temperature for 1 h. The HRP signals were detected using ECL and visualized using X-OMAT-Blue film (Kodak, Rochester, NY, USA).

Cell cycle analysis by flow cytometry. Cell lines were cultured in six-well plates for 24 h and then treated with Ad5-TFPI-2 or Ad5-GFP (MOI 50). After 72 h, cells were collected,

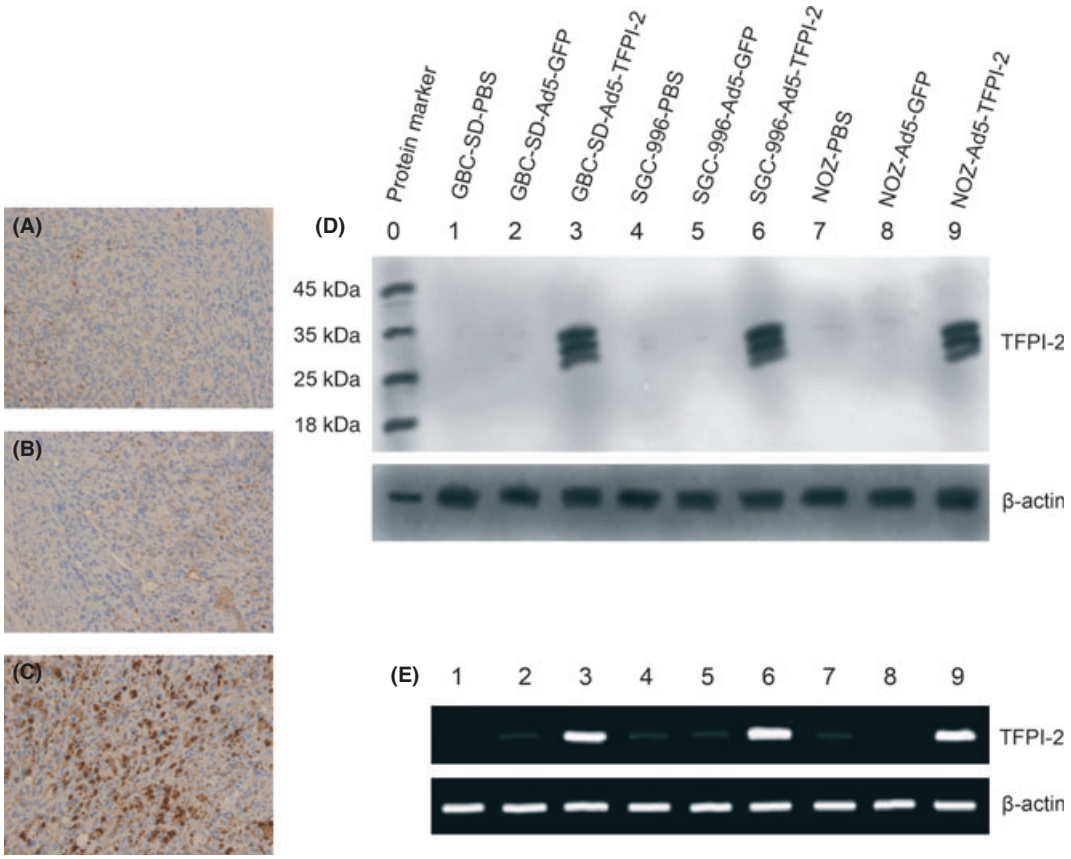

Fig. 2. Adenovirus-mediated gene transfer of tissue factor pathway inhibitor-2 (Ad5-TFPI-2) restored TFPI-2 expression in gallbladder carcinoma (GBC) cell lines and xenograft tumors. (A) TFPI-2 was weakly expressed in xenograft tumor tissues treated with phosphate-buffered saline (PBS) (×200). (B) TFPI-2 was weakly expressed in xenograft tumor tissues treated with Ad5-GFP. (C) TFPI-2 expression was detected in xenograft tumor tissues treated with Ad5-TFPI-2. (D) Western blotting analysis showed that TFPI-2 protein was detected in Ad5-TFPI-2 group (lane 3, 6, 9). All TFPI-2 containing fractions consisted of three differentially glycosylated molecular weight species (33, 31 and 27 kd). But none was observed in control groups (lane 1, 2, 4, 5, 7, 8). (E) The polymerase chain reaction (PCR) products of TFPI-2 and β-actin were 194 and 161 bp in length, respectively. TFPI-2 mRNA was detected in Ad5-TFPI-2 group (lane 3, 6, 9), whereas, the target bands were hardly seen in PBS group (lane 1, 4, 7) or Ad5-GFP group (lane 2, 5, 8). The lanes were designated the same as (D).

washed twice with PBS, and then fixed in 70% ethanol at 4°C for 30 min. After fixation, cells were washed twice with PBS and stained by PI (Roche, Basel, Switzerland) and R Nase A solution (Roche) at 37°C for 1 h. DNA contents of samples were examined by flow cytometry (Beckman Cytomics TM-7 FC500, Beckman Coulter, Brea, CA, USA). Cylchred version 1.0.2 software (Cardiff University, Wales, UK) was used to analyze cell cycle distribution.

Statistical analysis. All statistical analyses were performed using SPSS 11.0. The data were presented as mean ± SD. One-Way ANOVA or chi-sqaured test was used for the comparison analysis among three groups when appropriate. $P < 0.05$ was considered statistically significant. All assays were performed at least three times.

Results

Expression of TFPI-2 was downregulated in GBC tissues. Positive immunostaining of TFPI-2 protein was mainly located in the cytoplasm of epithelial cells for normal tissues or polyp tissues, occasionally in the cytoplasm of cancer cells and macrophages for GBC tissues (Fig. 1). As shown in Table 1, the positive rate of TFPI-2 expression was only 11.8% (4/34) in GBC cases, whereas it was 79.2% (19/24) in normal cases and 45.8% (11/24) in GBP cases ($P = 0.000$, chi-squared test). Furthermore, down-regulation of TFPI-2 was found to correlate with high UICC stages and lymph node metastasis. Of four cases of GBC patients with positive TFPI-2 expression, two were diagnosed with stage I and the other two with stage II. By contrast, none of the cases with stage III or IV were TFPI-2 staining positive. As for 16 cases with lymph nodes metastasis, TFPI-2 staining was all undetectable.

Ad5-TFPI-2 restored the expression of TFPI-2 in GBC cell lines and xenograft tumors. Ad5-TFPI-2 was constructed to restore the expression of TFPI-2 in GBC cell lines and xenograft tumors. As shown in Figure 2, the expression of TFPI-2 mRNA and protein was detected in three cell lines treated with Ad5-TFPI-2, but was absent or weak in the cells treated with Ad5-GFP or PBS, indicating that Ad5-TFPI-2 restored the expression of TFPI-2 in those cell lines successfully. Similarly, TFPI-2 immunoreactivity was strong in xenograft tumors treated with Ad5-TFPI-2, whereas it was weak in those treated with Ad5-GFP and PBS (Fig. 2).

TFPI-2 inhibited the growth of GBC *in vitro* and *in vivo*. To determine the inhibitory activities of TFPI-2 against GBC cell growth, the cell viability was examined by the WST-1 assay. As shown in Figure 3, Ad5-TFPI-2 exhibited a significant dose-dependent anti-proliferative activity in all three GBC cell lines ($P = 0.000$, one-way ANOVA). Meanwhile, Ad5-GFP showed cytotoxic effects against cell viability at MOI of 100, 500, and there was no significance in the total survival (including all MOI) between the Ad5-GFP group and PBS group ($P > 0.05$). For the *in vivo* study, xenograft tumors treated with Ad5-TFPI-2 grew much more slowly than those treated with Ad5-GFP or PBS. The means of tumor volumes in Ad5-TFPI-2, Ad5-GFP and PBS groups were 1.168 ± 0.105, 2.400 ± 0.420, 2.481 ± 0.257 cm^3, respectively, ($P = 0.000$, one-way ANOVA).

Enhanced TFPI-2 expression correlated with high apoptosis in GBC cell lines and xenograft tumors. The apoptosis index in GBC cell lines and xenograft tumors was correspondingly evaluated by Annexin V/PI staining and TUNEL assay. As shown in Table 2 and Figure 4(A), the percentages of apoptotic cells in Ad5-TFPI-2-treated group were significantly higher than those in control groups ($P = 0.000$, one-way ANOVA). For GBC-SD cells, the percentages of apoptotic cells in Ad5-TFPI-2 group, Ad5-GFP group and PBS group were 45.13 ± 1.70%, 11.67 ± 1.78% and 8.27 ± 1.89%, respectively. For SGC-996 cells, they were 33.70 ± 2.95%, 12.73 ± 2.31% and 7.87 ± 0.35%. For NOZ cells, they were 30.43 ± 2.17%, 11.90 ± 2.43% and 7.57 ± 1.21%. The TUNEL assay showed that Ad5-TFPI-2 induced more severe apoptosis in cancer cells than Ad5-GFP and PBS did (Fig. 4B–D), and there was no significant difference in the apoptosis index between the latter groups ($P > 0.05$). Apoptotic cells were counted under ×5 high-power fields. The

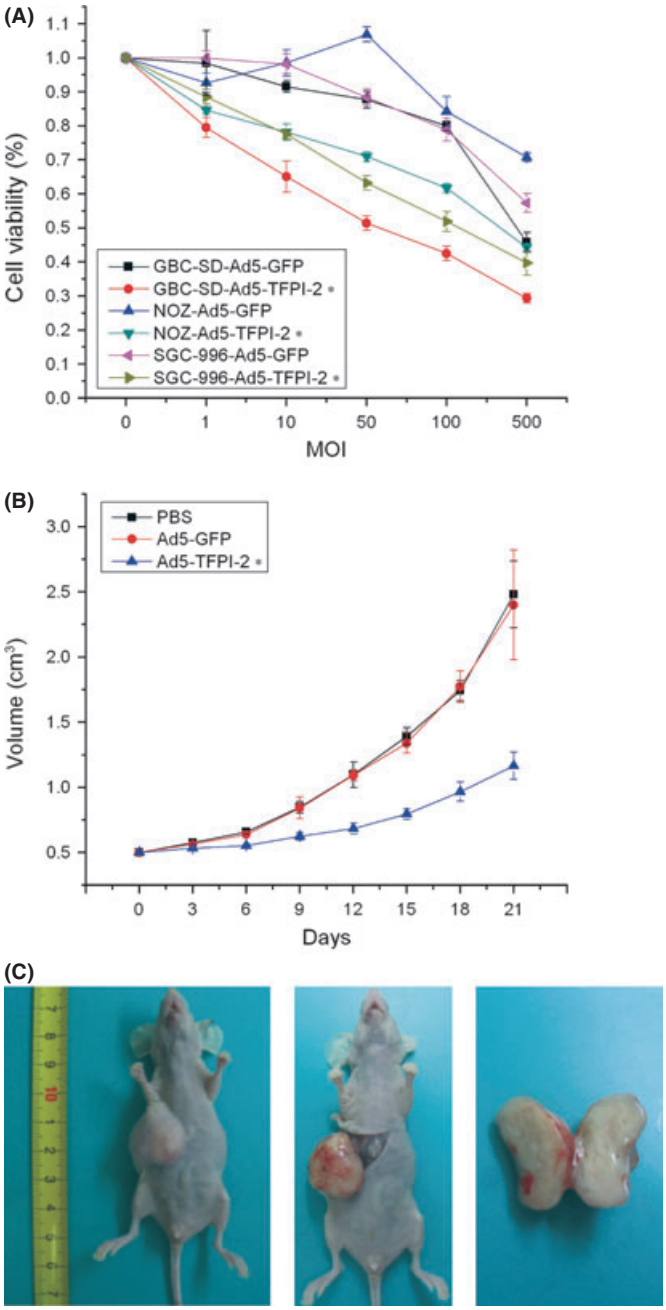

Fig. 3. Adenovirus-mediated gene transfer of tissue factor pathway inhibitor-2 (Ad5-TFPI-2) inhibited gallbladder carcinoma (GBC) growth *in vitro* and *in vivo*. Asterisk (*) indicates significant difference. (A) Cell viability was measured by WST-1 assay. Ad5-TFPI-2 exhibited a dose-dependent anti-proliferative activity against all three GBC cell lines. Cell proliferation was significantly inhibited in Ad5-TFPI-2 group when compared to control groups ($P = 0.000$, one-way ANOVA). (B) Volumes of xenograft tumors (cm^3) were measured after death. Volume = length × $width^2/2$. Tumor volumes in Ad5-TFPI-2 group were much smaller than those of control groups ($P = 0.000$, one-way ANOVA). (C) Xenograft tumors were collected and the volumes were measured.

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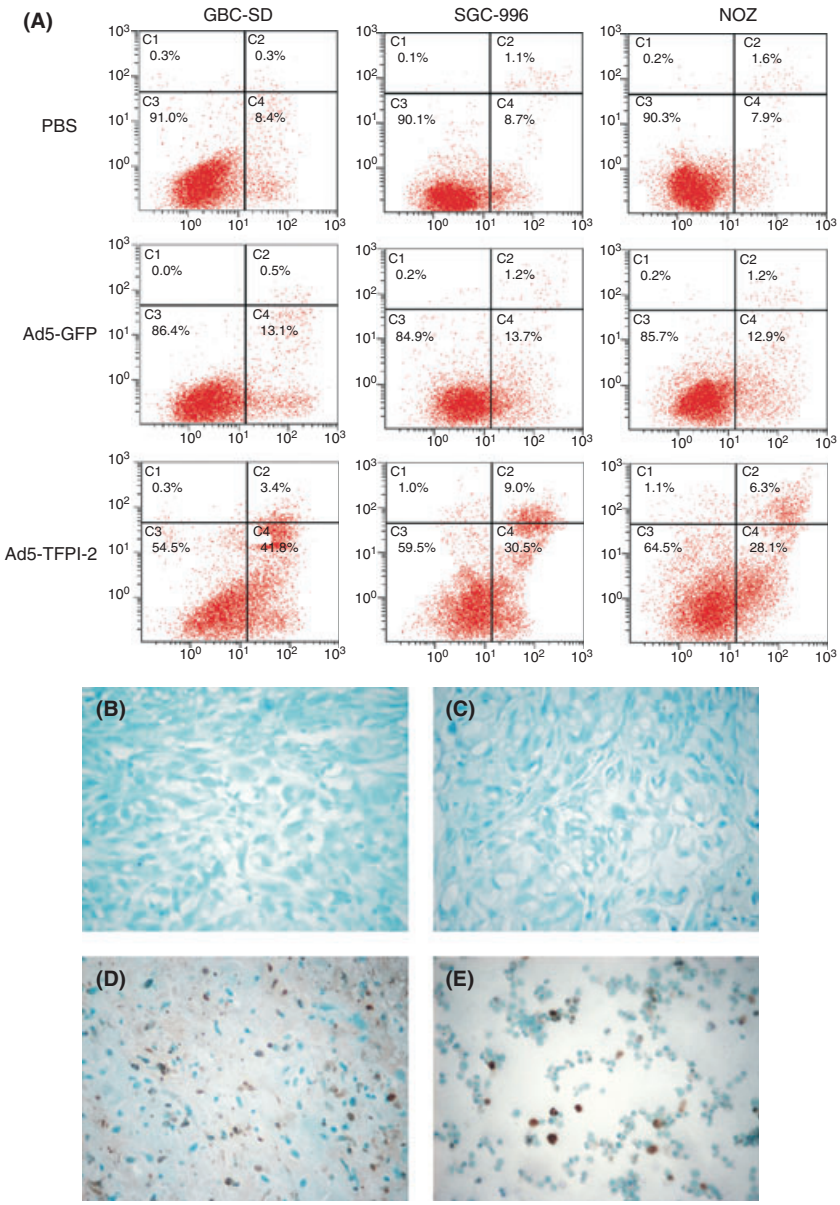

Fig. 4. Apoptosis in gallbladder carcinoma (GBC) cell lines and tissues was evaluated by Annexin V-APC/PI assay (A) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (B,C,D), respectively. (A) Adenovirus-mediated gene transfer of tissue factor pathway inhibitor-2 (Ad5-TFPI-2) induced apoptosis in all three GBC cell lines ($P = 0.000$). Cell apoptosis was examined by using Annexin V and propidium iodide (PI) staining. *X*-axis represents Annexin V fluorescence intensity and *Y*-axis represents PI fluorescence intensity. Cells that stain positive for Annexin V and negative for PI are undergoing apoptosis (lower right). Cells that stain positive for both Annexin V and PI are either in the end stage of apoptosis, undergoing necrosis or already dead (upper right). (B) Phosphate-buffered saline (PBS) group (×400). (C) Ad5-GFP group. (D) Ad5-TFPI-2 group. (E) Positive control (a mixture of HL60 cells incubated with 0.5 μg/mL actinomycin D for 19 h to induce apoptosis, provided by Merck). A dark brown 3,3′-diaminobenzidine tetrachloride (DAB) signal indicates positive staining while shades of blue to green signifies a non-reactive cell. Counterstaining with methyl green aids in the morphological evaluation and characterization of normal and apoptotic cells.

means of total apoptotic cells per field were 22.60 ± 2.70, 1.80 ± 0.84, 2.40 ± 0.89 in Ad5-TFPI-2, PBS and Ad5-GFP groups, respectively, ($P = 0.000$, one-way ANOVA).

TFPI-2 induced cell apoptosis by triggering cytochrome *c* release, upregulating pro-apoptotic factors and downregulating anti-apoptotic factor. After the high expression of TFPI-2 was found in both GBC cell lines and tissues with respect to apoptosis, we further investigated the mechanism underlying apoptosis induction in this context. We examined the variations of cytochrome *c*, Bax, Bcl-2, caspase-3, caspase-9 and p53 in protein level after transfection with Ad5-TFPI-2 by the western blotting assay. It turned out that the expression of cytochrome *c* in cytosol was significantly increased, and the expressions of cleaved caspase-3, -9 and Bax were also increased, whereas the expression of Bcl-2 was decreased (Fig. 5). In addition, no significant change in the expression of total p53 was observed.

Cell cycle analysis and the expressions of RB/pRB, p21, p27 in GBC-SD cells. As shown in Table 3, for three GBC cell lines, there was no significant difference in cell cycle distribution among three groups ($P > 0.05$, one-way ANOVA). In addition, no alteration was found in the expression of p21, p27 or RB/pRB (Fig. 5).

Discussion

This study was aimed to evaluate the anti-tumor effects of TFPI-2 on GBC. First, we examined the expression of

Table 2. Percentages of apoptotic cells in gallbladder carcinoma (GBC) cell lines

Cell lines	Apoptosis index		
	Ad5-TFPI-2 (%)	Ad5-GFP (%)	PBS (%)
GBC-SD	45.13 ± 1.70*	11.67 ± 1.78	8.27 ± 1.89
SGC-996	33.70 ± 2.95*	12.73 ± 2.31	7.87 ± 0.35
NOZ	30.43 ± 2.17*	11.90 ± 2.43	7.57 ± 1.21

Adenovirus-mediated gene transfer of tissue factor pathway inhibitor-2 (Ad5-TFPI-2) apparently induced apoptosis in all three groups ($P = 0.000$, one-way ANOVA). *Indicates significant difference.

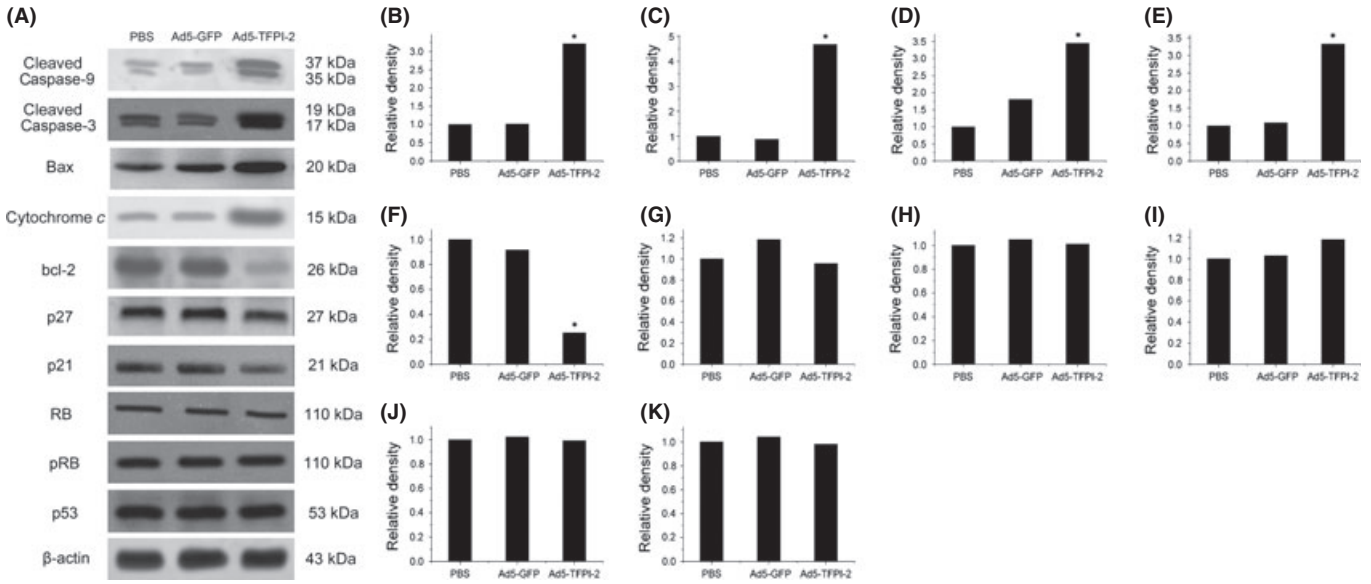

Fig. 5. The expressions of apoptosis-related and cell-cycle-related proteins in GBC-SD cells were examined by western blotting. (A) The expressions of cytochrome *c*, Bax, cleaved caspase-3, -9 were increased after treatment with adenovirus-mediated gene transfer of tissue factor pathway inhibitor-2 (Ad5-TFPI-2), whereas the expression of Bcl-2 was decreased. In addition, the expression of total p53 protein was not significantly changed for all three groups after treatment with Ad5-TFPI-2. The expressions of RB/pRB, p21 and p27 were also examined, and no significant alteration was observed. The optical density of target bands and control bands was quantified using Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). The density ratios of target bands to control bands were used to analyze the difference among each band. The numerical values were shown as gray histograms. (B–K) Relative density of cleaved caspase-9, cleaved caspase-3, Bax, cytochrome *c*, Bcl-2, p27, p21, RB, pRB, p53. Asterisk (*) indicates significant difference.

TFPI-2 in GBC cell lines and tissues. Consistent with the studies on gastric, pancreatic, hepatic, esophageal, renal and colon cancer and gliomas,[13,15] downregulation or absence of TFPI-2 protein was found in GBC tissues and cell lines, due to the lack of gene expression. Furthermore, TFPI-2 expression negatively correlated with the GBC stages. More notably, TFPI-2 protein was less expressed in GBP tissues than in normal tissues. There were some characteristic biological changes of epithelial cells in GBP tissues, as suggested by our study that unlike simple columnar epithelium in normal tissues, increased simple cuboidal cells (Fig. S1), epithelial dysplasia (Fig. S2), intestinal metaplasia (Fig. S3) and pseudopyloric metaplasia (Fig. S4) were found in GBP tissues. Besides, mucosal atrophy was frequently observed in GBP tissues due to chronic inflammation (Fig. S5). Such benign lesions may result from altered gene expression. Our study illustrated that the decreased TFPI-2 was likely to be involved in the altered biological behavior from normal cells to benign and/or to malignant tumor cells. In cancer cells, the loss of the locus for TFPI-2 gene in chromosome 7q region,[16] aberrant methylation of TFPI-2 gene promoter[15] and aberrantly spliced variant of TFPI-2[17] may result in the lack of TFPI-2 in these cells. However, for benign lesions, its regulation mechanisms and functions may be different. Based on the facts that under physiological and pathological conditions, TFPI-2 may exert various biological functions such as cell proliferation,[18] angiogenesis[19] and inflammation,[20] we considered that decreased TFPI-2 expression may contribute to abnormal proliferation, angiogenesis, and inflammation in GBP tissues. Certainly, the molecular mechanisms underlying the regulation of TFPI-2 in normal, GBP and GBC tissues remains to be determined. Additionally, our data suggested that TFPI-2 may act as a tumor suppressor during GBC tumorigenesis, and, as a secreted protein, TFPI-2 can be secreted into ECM, which may provide a new auxiliary way in the diagnosis of GBC by examining its level in bile.

Subsequently, we successfully restored TFPI-2 expression in GBC-SD, SGC-996, NOZ cells and xenograft tumors through the use of Ad5-TFPI-2. It has been reported that restoration of TFPI-2 expression can inhibit tumor growth in several cancers, including meningioma, fibrosarcoma, gliomas and nasopharyngeal carcinoma.[21–24] Our data showed that Ad5-TFPI-2 significantly inhibited the growth of GBC cell lines and xenograft tumor. We also found adenoviral vector itself had a dose-dependent cytotoxic effect *in vitro*, as the amount of applied virus highly correlated with the level of cell death, which was consistent with other studies.[25,26] Once the MOI reached 100, Ad5-GFP also showed cytotoxic effects against cell viability. However, we did not find any difference in xenograft tumor volume between Ad5-GFP and PBS groups. As the MOI *in vivo* was difficult to define, it was likely that the Ad5-GFP transfected into cells *in vivo*

Table 3. Cell cycle analysis after treatments

Cell line	Treatments	Cell cycle distribution (G1, S, G2)		
		G1 phase (%)	S phase (%)	G2 phase (%)
GBC-SD	Ad5-TFPI-2	57.15 ± 4.18	21.22 ± 1.61	21.63 ± 5.16
	Ad5-GFP	56.92 ± 3.37	24.54 ± 0.93	18.54 ± 3.6
	PBS	54.63 ± 3.18	29.80 ± 2.60	15.57 ± 1.11
SGC-996	Ad5-TFPI-2	57.68 ± 4.69	27.64 ± 3.03	14.68 ± 2.28
	Ad5-GFP	60.26 ± 4.26	24.75 ± 2.55	14.99 ± 3.85
	PBS	60.37 ± 4.65	22.47 ± 2.66	17.16 ± 2.13
NOZ	Ad5-TFPI-2	56.65 ± 3.33	29.34 ± 3.53	14.01 ± 1.68
	Ad5-GFP	56.87 ± 2.62	28.54 ± 1.33	14.59 ± 1.31
	PBS	51.84 ± 3.38	27.70 ± 1.47	20.46 ± 1.91

Adenovirus-mediated gene transfer of tissue factor pathway inhibitor-2 (Ad5-TFPI-2) did not change cell cycle distribution of all three cell lines ($P > 0.05$).

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may not reach MOI 100 and the complexity *in vivo* may reduce the toxicity of adenovirus itself. On the other hand, the TUNEL assay showed that Ad5-TFPI-2 significantly induced apoptosis in tumor tissues and there was no significant difference in the apoptosis index between Ad5-GFP group and PBS group. It seemed that the apoptosis was induced by the transfer of TFPI-2 gene, which also supported the results of xenograft tumor volume examination. Therefore, a precise MOI adjustment should be ensured to avoid cytotoxic effects of adenovirus itself when it is applied in preclinical studies or clinical trials.

Both FACS analysis and TUNEL assay for apoptosis showed increased apoptosis in cell lines and xenograft tumor tissues after TFPI-2 restoration. To further investigate underlying mechanisms for apoptosis, we examined the expressions of several apoptosis-related proteins in GBC-SD cells. We found that the expression of cytochrome *c* in cytosol was significantly increased, indicating that TFPI-2 was able to trigger cytochrome *c* release from mitochondria to cytosol. The release of cytochrome *c*, which is controlled by changes in the expression of Bax/Bcl-2 ratio[27] recruits and activates caspase-9, which eventually activates downstream caspases such as the major executioner caspase-3.[28,29] We therefore examined the expressions of the above proteins after treatments, and found that the expressions of Bax, cleaved caspase-3 and -9 were increased, whereas the expression of Bcl-2 was decreased. These data suggested that TFPI-2 could induce intrinsic apoptosis through promoting cytochrome *c* release. In addition, we also examined the status of p53, which is frequently mutated in GBC and plays an important role in the regulation of cell apoptosis as well as cell-cycle inhibition.[30] The western blotting analysis showed that the amount of total p53 was not increased. Taken together, we supposed that Ad5-TFPI-2 may induce the activation of intrinsic apoptosis pathway in a p53-independent manner in GBC-SD cells. However, why and how TFPI-2 can induce intrinsic apoptosis need to be further investigated. It has been demonstrated that the intrinsic apoptosis pathway can be activated by extrinsic apoptosis pathway and caspase-8 is able to mediate crosstalk between the two pathways.[31,32] Since we did not examine the status of caspase-8, Bid, or death receptors such as Fas/FasL or TRAIL/TRAILR in this study, it is difficult to know whether TFPI-2-induced intrinsic apoptosis would be caused by death receptors or other apoptotic signaling. Thus, there leaves the possibility that extrinsic apoptotic pathway would also be involved in TFPI-2-induced apoptosis. Future studies on apoptotic signaling in GBC-SD cells will help to clarify this issue.

It has been reported that TFPI-2 could also regulate cell proliferation through cell cycle, while transfer of TFPI-2 gene into GBC cell lines did not have an effect on the progress of cell cycle in the present study. The western blotting for RB/pRB, p21 and p27, all of which are important cell cycle regulators, further supported the result of cell cycle analysis. Cell cycle regulation by TFPI-2 was mainly found in normal cells,[33–35] and its ability to affect cell cycle may be cell type-specific. In malignant cells, TFPI-2 was not found to affect cell cycle status. These findings indicated that TFPI-2-induced tumor growth inhibition could be mainly caused by apoptosis induction rather than by cell cycle regulation.

In this study, we used adenovirus-mediated gene transfer, since recombinant adenoviral vectors represent one of the best gene transfer platforms due to their ability to efficiently transduce a wide range of quiescent and proliferating cell types from various tissues and species and their relative safety. Our previous study also demonstrated that adenovirus was a good gene transfer platform for GBC.[36] The precise intratumor injection of Ad5-TFPI-2 could be performed by percutaneous route under ultrasound or computed tomography (CT) guidance. Additionally, the toxicity of Ad5-TFPI-2 should be supervised.

Our data strongly suggested that TFPI-2 acts as a tumor suppressor in GBC and could be a promising tool in gene therapy for GBC in the future. When it comes to application in clinical settings, restoration of TFPI-2 may be another chance for patients with advanced GBC insensitive to chemotherapy/radiotherapy or untreatable by surgery. In fact, a clear therapeutic effect of adenoviral gene therapy on cancer has not yet been achieved and there are no clinical trials about adenovirus-mediated gene therapy for GBC currently. More evidence for wider clinical trials are needed to improve its safety and efficacy. Though the role of TFPI-2 has been initially investigated in this study, there are still some issues to be solved and clarified in future studies. Better understanding of the molecular mechanisms underlying the regulation of TFPI-2 will pave the way for the development of new diagnostic and therapeutic strategies for patients with GBC.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Misra S, Chaturvedi A, Misra NC, Sharma ID. Carcinoma of the gallbladder. *Lancet Oncol* 2003; **4**: 167–76.
2 Eslick GD. Epidemiology of gallbladder cancer. *Gastroenterol Clin North Am* 2010; **39**: 307–30, ix.
3 de Aretxabala X, Roa I, Berrios M *et al.* Chemoradiotherapy in gallbladder cancer. *J Surg Oncol* 2006; **93**: 699–704.
4 Gourgiotis S, Kocher HM, Solaini L, Yarollahi A, Tsiambas E, Salemis NS. Gallbladder cancer. *Am J Surg* 2008; **196**: 252–64.
5 Saetta A, Lazaris AC, Michalopoulos NV, Davaris PS. Genetic alterations involved in the development of gallbladder carcinomas from Greek patients. *Hepatogastroenterology* 2001; **48**: 1284–8.
6 Kim YT, Kim J, Jang YH *et al.* Genetic alterations in gallbladder adenoma, dysplasia and carcinoma. *Cancer Lett* 2001; **169**: 59–68.
7 Masuhara S, Kasuya K, Aoki T, Yoshimatsu A, Tsuchida A, Koyanagi Y. Relation between K-ras codon 12 mutation and p53 protein overexpression in gallbladder cancer and biliary ductal epithelia in patients with pancreaticobiliary maljunction. *J Hepatobiliary Pancreat Surg* 2000; **7**: 198–205.
8 Quan ZW, Wu K, Wang J, Shi W, Zhang Z, Merrell RC. Association of p53, p16, and vascular endothelial growth factor protein expressions with the prognosis and metastasis of gallbladder cancer. *J Am Coll Surg* 2001; **193**: 380–3.
9 Puhalla H, Wrba F, Kandioler D *et al.* Expression of p21(Wafl/Cip1), p57 (Kip2) and HER2/neu in patients with gallbladder cancer. *Anticancer Res* 2007; **27**: 1679–84.
10 Chand HS, Foster DC, Kisiel W. Structure, function and biology of tissue factor pathway inhibitor-2. *Thromb Haemost* 2005; **94**: 1122–30.

11 Petersen LC, Sprecher CA, Foster DC, Blumberg H, Hamamoto T, Kisiel W. Inhibitory properties of a novel human Kunitz-type protease inhibitor homologous to tissue factor pathway inhibitor. *Biochemistry* 1996; **35**: 266–72.
12 Herman MP, Sukhova GK, Kisiel W *et al.* Tissue factor pathway inhibitor-2 is a novel inhibitor of matrix metalloproteinases with implications for atherosclerosis. *J Clin Invest* 2001; **107**: 1117–26.
13 Wojtukiewicz MZ, Sierko E, Zimnoch L, Kozlowski L, Kisiel W. Immunohistochemical localization of tissue factor pathway inhibitor-2 in human tumor tissue. *Thromb Haemost* 2003; **90**: 140–6.
14 Sierko E, Wojtukiewicz MZ, Kisiel W. The role of tissue factor pathway inhibitor-2 in cancer biology. *Semin Thromb Hemost* 2007; **33**: 653–9.
15 Wong CM, Ng YL, Lee JM *et al.* Tissue factor pathway inhibitor-2 as a frequently silenced tumor suppressor gene in hepatocellular carcinoma. *Hepatology* 2007; **45**: 1129–38.
16 Sell SM, Tullis C, Strancner D, Song CY, Gewin J. Minimal interval defined on 7q in uterine leiomyoma. *Cancer Genet Cytogenet* 2005; **157**: 67–9.
17 Kempaiah P, Chand HS, Kisiel W. Identification of a human TFPI-2 splice variant that is upregulated in human tumor tissues. *Mol Cancer* 2007; **6**: 20.
18 Ma S, Chan YP, Kwan PS *et al.* MicroRNA-616 induces androgen-independent growth of prostate cancer cells by suppressing expression of tissue factor pathway inhibitor TFPI-2. *Cancer Res* 2011; **71**: 583–92.
19 Ivanciu L, Gerard RD, Tang H, Lupu F, Lupu C. Adenovirus-mediated expression of tissue factor pathway inhibitor-2 inhibits endothelial cell migration and angiogenesis. *Arterioscler Thromb Vasc Biol* 2007; **27**: 310–6.
20 Hisaka T, Lardeux B, Lamireau T *et al.* Expression of tissue factor pathway inhibitor-2 in murine and human liver regulation during inflammation. *Thromb Haemost* 2004; **91**: 569–75.
21 Kondraganti S, Gondi CS, Gujrati M *et al.* Restoration of tissue factor pathway inhibitor inhibits invasion and tumor growth *in vitro* and *in vivo* in a malignant meningioma cell line. *Int J Oncol* 2006; **29**: 25–32.
22 Kempaiah P, Kisiel W. Human tissue factor pathway inhibitor-2 induces caspase-mediated apoptosis in a human fibrosarcoma cell line. *Apoptosis* 2008; **13**: 702–15.
23 Tasiou A, Konduri SD, Yanamandra N *et al.* A novel role of tissue factor pathway inhibitor-2 in apoptosis of malignant human gliomas. *Int J Oncol* 2001; **19**: 591–7.
24 Wang S, Xiao X, Zhou X *et al.* TFPI-2 is a putative tumor suppressor gene frequently inactivated by promoter hypermethylation in nasopharyngeal carcinoma. *BMC Cancer* 2010; **10**: 617.
25 Müller J, Thirion C, Pfaffl MW. Electric cell-substrate impedance sensing (ECIS) based real-time measurement of titer dependent cytotoxicity induced by adenoviral vectors in an IPI-2I cell culture model. *Biosens Bioelectron* 2011; **26**: 2000–5.
26 Schierack P, Nordhoff M, Pollmann M *et al.* Characterization of a porcine intestinal epithelial cell line for *in vitro* studies of microbial pathogenesis in swine. *Histochem Cell Biol* 2006; **125**: 293–305.
27 Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**: 1309–12.
28 Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. *Nat Cell Biol* 2001; **3**: E255–63.
29 Ow YP, Green DR, Hao Z, Mak TW. Cytochrome c: functions beyond respiration. *Nat Rev Mol Cell Biol* 2008; **9**: 532–42.
30 Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. *Oncogene* 2005; **24**: 2899–908.
31 Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; **94**: 491–501.
32 Roy S, Nicholson DW. Cross-talk in cell death signaling. *J Exp Med* 2000; **192**: 21–6.
33 Zhao B, Luo X, Shi H, Ma D. Tissue factor pathway inhibitor-2 is downregulated by ox-LDL and inhibits ox-LDL induced vascular smooth muscle cells proliferation and migration. *Thromb Res* 2011; **128**: 179–85.
34 Shibuya M, Okamoto H, Nozawa T *et al.* Proteomic and transcriptomic analyses of retinal pigment epithelial cells exposed to REF-1/TFPI-2. *Invest Ophthalmol Vis Sci* 2007; **48**: 516–21.
35 Shinoda E, Yui Y, Hattori R *et al.* Tissue factor pathway inhibitor-2 is a novel mitogen for vascular smooth muscle cells. *J Biol Chem* 1999; **274**: 5379–84.
36 Jia J, Li S, Gong W, Ding J, Fang C, Quan Z. mda-7/IL-24 induces apoptosis in human GBC-SD gallbladder carcinoma cells via mitochondrial apoptotic pathway. *Oncol Rep* 2011; **25**: 195–201.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alteration from simple columnar cells to simple cuboidal cells.

Fig. S2. Epithelial dysplasia.

Fig. S3. Intestinal metaplasia.

Fig. S4. Pseudopyloric metaplasia.

Fig. S5. Mucosal atrophy.

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