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Methylation of *TFPI-2* is an early event of esophageal carcinogenesis

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Abstract

Aims—To explore the epigenetic changes and the function of *TFPI-2* in esophageal cancer.

Materials & methods—Nine esophageal cancer cell lines, nine normal esophageal mucosa, 60 esophageal dysplasia and 106 advanced esophageal cancer samples were included in this study. *TFPI-2* methylation was examined by methylation-specific PCR. *TFPI-2* expression was evaluated by immunohistochemistry in tissue samples. The effect of *TFPI-2* on proliferation, apoptosis, invasion and migration was analyzed by colony formation assay, western blot assay, transwell assay and flow cytometric analysis.

Results—*TFPI-2* expression was regulated by promoter region hypermethylation in human esophageal cancer cell lines, and *TFPI-2* expression is inversely correlated with methylation in primary cancer. Methylation was found in 28.2, 33.3 and 33.3% of grade 1, 2 and 3 esophageal dysplasia, and 67% of primary esophageal cancer, but no methylation was found in normal mucosa. Methylation is significantly related to tumor differentiation. Inhibition of invasion, migration, colony formation and proliferation, and induction of apoptosis occurred with the restoration of *TFPI-2* expression in the KYSE70 cell line.

Conclusion—*TFPI-2* is frequently methylated in esophageal cancer with a progression tendency. *TFPI-2* is a potential tumor suppressor in esophageal cancer.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Keywords

carcinogenesis; DNA methylation; dysplasia; esophageal cancer; *TFPI-2*

Esophageal cancer is the eighth most commonly occurring cancer and the sixth leading cause of cancer death in the world [1–3]. Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are the two major types of esophageal cancer. ESCC is the most frequent type of histology in China, whereas EAC is more common type of esophageal cancer in western countries [4,5]. EAC is highly associated with obesity and gastroesophageal reflux disease. Patients with gastroesophageal reflux disease have an increased risk for developing intestinal metaplasia at the lower part of esophagus (Barrett's esophagus), which is thought to be a precursor lesion of EAC [6,7]. Tobacco and alcohol abuse may increase esophageal cancer risk, especially in ESCC [3]. The development of human ESCC, on the other hand, occurs when squamous epithelial cells change through grade 1, grade 2 and grade 3 of dysplasia, carcinoma *in situ* to advanced ESCC [8]. Despite recent improvements in diagnosis and treatment of esophageal cancer, the overall survival rate remains disappointingly low. Thus, understanding the mechanism of esophageal cancer is desperately needed.

TFPI-2 is also known as matrix-associated serine protease inhibitor and placental protein 5 [9,10]. Reduced expression of *TFPI-2* has been found in several cancers, has been associated with tumor invasion and metastasis, and has been frequently reported to be methylated in different stages of invasive cancer [11–14]. In this study, we examined esophageal cancer cell lines, normal esophageal mucosa, various grades of esophageal dysplasia and primary esophageal cancer tissues to explore the possibility of *TFPI-2* methylation as an early detection marker as well as to understand the effect of *TFPI-2* on esophageal carcinogenesis.

Material & methods

Human tissue samples & cell lines

In this study, 106 cases of primary esophageal cancer tissues were examined. The median age of patients was 60 years old (range: 33–77 years) and the ratio of male:female was 2.4:1. Snap frozen fresh tissue samples were collected by surgery resection. All samples were classified by TNM (UICC 2009) staging, and included one case of stage I, 45 cases of stage II, 55 cases of stage III and five cases of stage IV. Matched adjacent tissues were available in 37 cases from the paraffin blocks of the 106 primary cancer samples. As for the esophageal dysplasia tissues, 39 cases consisted of low-grade dysplasia (ED1), 12 were cases of intermediate-grade dysplasia (ED2) and nine were cases of high-grade dysplasia (ED3) were collected as paraffin-embedded samples. Nine cases of normal esophageal epithelia were collected by endoscopy biopsy. All of the above samples were collected from the Chinese People's Liberation Army General Hospital (Beijing, China) under the guidelines approved by Chinese People's Liberation Army General Hospital's institutional review board.

Nine esophageal cancer cell lines (TE3, TE10, YSE2, KYSE30, KYSE70, KYSE140, KYSE150, KYSE450 and KYSE510) were used in this study. All esophageal cancer cell lines were previously established from primary esophageal cancer, and maintained in 90% Gibco® RPMI media 1640 (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum. Cells were passaged 1:3 once total confluence ($\sim 10^6$ cells) was reached on a 75 cm² culture flask (NEST Biotechnology, China).

5-aza-2'-deoxycytidine treatment

Esophageal cancer cell lines (TE3, TE10, YSE2, KYSE30, KYSE70, KYSE140, KYSE150, KYSE450 and KYSE510) were split to a low density (30% confluence) at 12 h before treatment. Cells were treated with 5-aza-2'-deoxycytidine (DAC) (Sigma, MO, USA) at a concentration of 2 μ M. Growth medium, conditioned with DAC at 2 μ M, was exchanged every 24 h for a total of 96 h of treatment. At the end of the treatment course, RNA was extracted from the cells as described below.

RNA isolation & semiquantitative RT-PCR

Total RNA was isolated by Trizol reagent (Life Technologies, MD, USA). Agarose gel (1%) electrophoresis and spectrophotometric analysis (A260:280 nm ratio) were used to evaluate RNA quality and quantity. RNA was stored at -80°C prior to use. First strand cDNA was synthesized according to the manufacturer's instructions; 5 μ g of the total RNA was used to synthesize first strand cDNA with random six-mer primers using a Superscript III-reverse transcriptase kit (Invitrogen). Following first strand synthesis, the reaction mixture was diluted to 100 μ l with water. Subsequently, 2.5 μ l of diluted cDNA mixture was used for PCR amplification in a final 25 μ l reaction volume. PCR amplification of *TFPI-2* was carried out using the primers: 5'-gggcctactctcgttac-3' (forward) and 5'-cacactggctgcccacactc-3' (reverse). The primer set for the *TFPI-2* gene was designed to span intronic sequences between adjacent exons in order to control for genomic DNA contamination. A total of 32 cycles of amplification was performed for each of the RT-PCR experiments. As an internal control, GAPDH was amplified with 25 cycles to ensure cDNA quality and quantity for each RT-PCR. Amplified products were analyzed on 1.5% agarose gels.

DNA extraction & bisulfite modification

Biopsy specimens were snap-frozen prior to DNA extraction. Paraffin-embedded tissue samples were sectioned at 10 μ m, and four–six tissue sections were deparaffinized in xylene and washed twice with 100% ice-cold ethanol. The deparaffinized tissue sections, snap-frozen fresh tissue, and biopsy samples were digested overnight with proteinase K. After treatment of proteinase K, genomic DNA from esophageal cancer cell lines, 106 cases of primary esophageal cancer, 60 cases of dysplasia, and nine cases of normal esophageal epithelia were all isolated by phenol-chloroform extraction. Then, DNA was precipitated in ethanol, later dissolved in low TE buffer, and then stored at -20°C . 2 μ g of genomic DNA was diluted in 50 μ l of water. The bisulfite treatment was carried out for 16 h at 50°C according to previously described procedures [15]. DNA samples were then purified with the Wizard DNA Clean-Up System (Promega, WI, USA), desulfonated with NaOH, and then precipitated with ethanol and resuspended in 20 μ l of water.

Nested PCR

Nested PCR was performed to facilitate the examination of *TFPI-2* methylation on paraffin-embedded dysplasia samples. The bisulfite-modified DNA was subjected to a first-stage PCR incorporating external primer sets. The PCR reaction was carried out in a total volume of 25 μ l with 1 unit of Taq Polymerase (Invitrogen), 25 pmol of external primer, 100 pmol dNTPs, 2.5 μ l 10 $\times$ PCR buffer, and 2 μ l of bisulfite-modified DNA. The external primer sequences were as follows: 5'-gtgtatgaattagttatttttaggttt-3' (nest-forward) and 5'-ctaaacaaactccraaaaaac-3' (nest-reverse). PCR conditions included an initial denaturation at 95°C for 5 min followed by 35 cycles of amplification ($95^{\circ}\text{C} \times 30$ s, $55^{\circ}\text{C} \times 30$ s, $72^{\circ}\text{C} \times 40$ s), and a final elongation step at 72°C for 5 min. PCR products were analyzed on a 2% agarose gel to confirm adequate template for subsequent second stage internal methylation-specific PCR (MSP).

Methylation-specific PCR

MSP primers were designed according to genomic sequences flanking the presumed transcriptional start sites. Primer sequences were oligo-synthesized (Invitrogen) to detect bisulfite-induced changes affecting unmethylated (U) and methylated (M) alleles. The MSP of *TFPI-2* was carried out using primers: 5'-atTTTTtaggttcgtt-tcggc-3' (M-sense); 5'-gcctaacgaaaaaaatacgcg-3' (M-antisense); 5'-ttagtatttttaggtttgttttggt-3' (U-sense) and 5'-aaaaacacctaacaaaaaaata-caca-3' (U-antisense). Each MSP reaction included approximately 200 ng of bisulfite-treated DNA, 25 pmol of each primer, 100 pmol dNTPs, 2.5 µl 10× PCR buffer, and 1 unit of Taq Polymerase (Invitrogen) in a final reaction volume of 25 µl. Cycle conditions were: 95°C × 5 min for 1 cycle; 35 cycles × (95°C × 30 s, 60°C × 30 s, 72°C × 30 s); 72°C × 5 min for 1 cycle. Each PCR assay included a positive control, using DNA treated *in vitro* with *SssI* methyltransferase (New England Biolabs, MA, USA), and a negative control, using normal human peripheral lymphocytes. MSP products were analyzed using a 2% agarose gel electrophoresis.

Bisulfite sequencing

KYSE30, KYSE70 and KYSE450 cell lines were sequenced by sodium bisulfite treatment in this study. Bisulfite-treated DNA was amplified using primers flanking the targeted regions, including the MSP amplified region and the transcriptional start site. Sequencing primers were as follows: 5'-GGGGTGATAGTTTTYGTGTA-3' (forward) and 5'-CRCCCAATACAAC-CTCCRTC-3' (reverse). The size of the PCR product is 249 bp (−107 bp to +142 bp). PCR cycle conditions were as follows: 95°C × 5 min for 1 cycle; 35 cycles × (95°C × 30 s, 55°C × 30 s, 72°C × 40 s); 72°C × 5 min for 1 cycle. PCR products were gel purified and cloned into pCR2.1 vectors according to the manufacturer's protocol (Invitrogen). Sequencing was performed as previously described [16].

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4-µm thick serial sections derived from formaldehyde-fixed paraffin blocks of esophageal cancer and paired adjacent tissue. After deparaffinization and rehydration, endogenous peroxidase activity was blocked for 30 min in methanol containing 0.3% hydrogen peroxide. After antigen retrieval, a cooling-off period of 20 min preceded the incubation of the primary antibody. In short, antigen retrieval was performed (45 min at 96°C) in target retrieval solution. The primary rabbit antibody (anti-TFPI-2 with 1/150 dilution; Abcam, MA, USA) was incubated overnight at 4°C. Thereafter, the catalyzed signal amplification system (ZSGB Biotechnology, Beijing, China) was used to detect TFPI-2 staining according to manufacturer's instructions.

The expression level of TFPI-2 was evaluated by both intensity and extent of the positive staining. The intensity of TFPI-2 expression was quantified using scores as follow: 0 = negative, 1 = weakly positive, 2 = moderately positive and 3 = strongly positive. The extent of TFPI-2 expression was quantified as percentage of positive staining areas in relation to whole tissue areas. The score standard is shown as follows: 0 = 0% reactivity, 1 = 1–10% reactivity, 2 = 11–50% reactivity, 3 = 51–80% reactivity and 4 points refers to samples with >80% reactivity. The final IHC score was determined by multiplying intensity score by extent score, with the minimum score of 0 and maximum score of 12 points. Therefore, IHC scores include: 10–12: strong TFPI-2 expression (+++); 7–9: intermediate TFPI-2 expression (++); 3–6: weak TFPI-2 expression (+); and 0–2: negative TFPI-2 expression (−). A score of 3 points or greater was considered positive for TFPI-2 expression.

A χ^2 test was applied to analyze the correlation between *TFPI-2* methylation and its staining.

Construction of TFPI-2 expression vector & transfection assay

Full-length *TFPI-2* cDNA (GenBank accession number NM_006528) was cloned by RT-PCR from cDNA derived from placenta into the pCMV6-AC vector (Origene Technology, MD, USA). The TFPI-2 expression vector was verified by DNA sequencing.

Transient transfection was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Colony formation assay

KYSE70 cells were grown in six-well culture plates 24 h before transfection. Cells were transfected with empty vector or TFPI-2 expression vector according to the manufacturer's instructions (Invitrogen). After 36 h, the cells were diluted and reseeded 1500 cells/well in six-well culture plates in triplicate. Growth medium, conditioned with G418 (Invitrogen) at 450 µg/ml, was exchanged every 24 h. After 14 days, the cells were fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet for visualization and counting.

Cell invasion assay

The effect of TFPI-2 on cell invasion was detected by the Transwell assay (COSTAR transwell, Corning Incorporated, MA, USA). KYSE70 cells were transfected with empty vector or TFPI-2 expression vector. 3×10^5 cells were suspended in 300 µl of serum-free Gibco RPMI Media 1640 and loaded onto the upper compartment of an invasion chamber containing a polycarbonate membrane with an 8 µm pore size which was coated with a layer of extracellular matrix (ECM; Matrigel™, BD, NJ, USA). After 48 h of incubation, the invasive cells migrated through the ECM layer to the complete medium in the lower compartment. The invasive cells were stained with crystal violet and the number of invaded cells were counted in three independent high powered fields ($\times 100$) readings with a light microscope. Statistical analysis was applied among the groups.

Analysis of cell migration

The effect of TFPI-2 on cell migration was detected by using the Transwell assay in the absence of the ECM layer. KYSE70 cells were transfected with empty vector or TFPI-2 expression vector. After 48 h, TFPI-2 forced expressed cells, empty vector transfected cells, or untransfected cells were harvested and suspended in the serum free Gibco RPMI media 1640. Cell suspensions were then placed into the upper well at a concentration of 1×10^4 cells/100 µl separately, while the complete medium with 15% fetal bovine serum was placed into the lower well (500 µl). The chamber was incubated for 4 h. Nonmigrated cells on the upper surface were scraped gently and washed out with PBS three times. KYSE70 cells migrated to the lower surface of the membrane were stained with crystal violet and counted in three independent high powered fields ($\times 100$) with light microscope. Statistical analysis was applied among groups.

Analysis of apoptosis & cell cycle

Early and late apoptosis was detected by Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (KeyGen Biotechnology, China). KYSE70 cells were transfected with empty vector or TFPI-2 expression vector according to manufacturer's instruction. Treated or untreated KYSE70 cells (2×10^5 cells/sample) were washed twice in cold PBS (pH 7.4), resuspended in 500 µl binding buffer with 5 µl of Annexin V-FITC and 5 µl PI. The samples were incubated for 15 min in the dark at room temperature and analyzed by flow cytometry (Beckman Coulter, CA, USA).

Cell cycle progression was examined by quantization of cellular DNA content after staining with PI (KeyGen Biotechnology). KYSE70 cells were transfected with empty vector or TFPI-2 expression vector according to manufacturers instruction. Treated or untreated KYSE70 cells were collected by centrifugation, suspended in PBS, and fixed in ice-cold 70% ethanol for 24 h at 4°C. Then cells were washed in PBS and incubated with 100 µl RNase A at 37°C for 30 min. After adding 400 µl PI, the samples were taken at 4°C in the dark until flow cytometric analysis. The percentage of cells in G0/1, S and G2/M phase was evaluated with ModFit LT software (Verity Software House, ME, USA).

Protein preparation & western blotting

KYSE70 cells were transfected with TFPI-2 expression vector or empty control vector. 48 h later, transfected cells were harvested and lysed in ice-cold Tris buffer (20 mmol/l Tris; pH 7.5) containing 137 mmol/l of NaCl, 2 mmol/l of EDTA, 1% Triton X, 10% glycerol, 50 mmol/l of NaF, 1 mmol/l of DTT, and a protease inhibitor cocktail (Roche Applied Science, IN, USA). 40 µg of cell lysate was loaded into each lane. The protein lysates were then separated by SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes (Hybond-P, Amersham). After blocking with 5% nonfat milk and 0.1% Tween-20 in Tris buffered saline, the membranes were incubated with rabbit antibodies of anti-TFPI-2 (Abcam, MA, USA), anticyclin D1 (Bioworld Technology, MN, USA), anti-c-Myc (Bioworld Technology), anti-MMP-2 (Bioworld Technology) or mouse antibody of anti-β-actin (Beyotime Biotechnology, China). β-actin antibody was used in a reprobing as a loading control. The blots were visualized using enhanced chemiluminescence (Pierce Bioscience, IL, USA).

Statistical analysis

Statistical analysis was carried out using χ^2 test and $p < 0.05$ was considered statistically significant.

Results

TFPI-2 was silenced by promoter region hypermethylation in esophageal cancer cell lines

To explore TFPI-2 expression in esophageal cancer, nine esophageal cancer cell lines were used to detect expression by semi-quantitative RT-PCR. TFPI-2 expression was shown in TE10, KYSE450 and weakly in TE3, YSE2, KYSE30 and KYSE140 cell lines. No expression was found in KYSE70, KYSE150 and KYSE510 cell lines (Figure 1a). *TFPI-2* promoter region methylation was examined by MSP. The promoter regions were completely methylated in KYSE70, KYSE150 and KYSE510 cell lines and partially methylated in TE3, YSE2, KYSE30 and KYSE140 cell lines. No methylation was found in TE10 and KYSE450 cell lines (Figure 1B). These results indicated that loss of TFPI-2 expression was correlated with promoter region methylation.

The results of bisulfite sequencing was shown in Figure 1C. The results correlated with our MSP findings with *TFPI-2* being completely methylated at the promoter in KYSE70, partially methylated in KYSE30 and unmethylated in KYSE450 cell lines. The mixed methylated and unmethylated clones in the KYSE30 cells may either represent the presence of both methylated and unmethylated alleles or the existence of both methylated and unmethylated clonal subpopulations within the cultured cells. These data indicate that these MSP primers are properly situated for methylation detection.

To further validate whether TFPI-2 expression was regulated by promoter region methylation, DAC treatment was applied in these esophageal cancer cell lines. DAC may induce re-expression of methylated genes as it is a DNA methylation transferase inhibitor

[17,18]. The expression of TFPI-2 was examined by semiquantitative RT-PCR before and after 96 h of DAC treatment. As shown in Figure 1A, no expression and complete methylation were found before DAC treatment in KYSE70, KYSE150 and KYSE510 cell lines, and re-expression of TFPI-2 appeared after DAC treatment in these cells. The cell lines of TE3, YSE2, KYSE30 and KYSE140, that had weakly expressed and partially methylated *TFPI-2*, had restoration of strong TFPI-2 expression after DAC treatment. In TE10 and KYSE450 cell lines in which TFPI-2 was strongly expressed and unmethylated, there were no expression changes before or after DAC treatment. These results demonstrate that TFPI-2 is silenced by promoter region methylation in certain esophageal cancer cell lines.

TFPI-2 was frequently methylated in esophageal early lesion & invasive cancer

TFPI-2 methylation was examined in 106 cases of primary esophageal cancer, 60 cases of different grades of dysplasia and nine cases of normal esophageal epithelia. As shown in Figure 2A1, 2A2 & 2A3, 67% (71 of 106) of primary esophageal cancer were methylated, 33.3% (three out of nine) of ED3, 33.3% (four out of 12) of ED2 and 28.2% (11 out of 39) of ED1 were methylated. But none of the nine normal esophageal mucosa was methylated. These results indicated *TFPI-2* was methylated in the early stage of esophageal carcinogenesis. To explore if *TFPI-2* methylation had a tendency to progress during esophageal carcinogenesis, χ^2 test was applied in this study. Because of limitations on sample size, ED2 and ED3 were merged together. As shown in Figure 2A4, the methylation frequency of *TFPI-2* progressively increased from normal esophageal epithelia to advanced cancer (χ^2 test, $p = 0.0001$).

The association of *TFPI-2* methylation and clinical factors were also analyzed in this study. As shown in Table 1, *TFPI-2* methylation is significantly related to tumor differentiation (χ^2 test, $p = 0.0323$). No association was found significantly between *TFPI-2* methylation and age, gender, smoking, alcohol, tumor size, stage or metastasis.

Loss of TFPI-2 expression was related to promoter region methylation in human primary esophageal cancer

To explore TFPI-2 expression in human esophageal cancer tissue, IHC staining was performed on 37 cases of available paired cancer and adjacent tissue samples. TFPI-2 staining was mainly located in the cytoplasm. Negative staining was detected in 24 cases of esophageal cancer and 13 cases of adjacent tissue (Figure 2B1 & 2B2). Positive staining was found in 13 cases of esophageal cancer and 24 cases of adjacent tissue (Figure 2B3 & 2B4). TFPI-2 expression is significantly difference in esophageal cancer and adjacent tissue (χ^2 test, $p = 0.0105$). *TFPI-2* methylation was also analyzed in these paired samples. Twenty seven cases were methylated and ten cases were unmethylated in cancer tissue. A total of 11 cases were methylated and 26 cases were unmethylated in 37 cases of adjacent tissue. The promoter region methylation is inversely associated with TFPI-2 staining (χ^2 test, $p = 0.0018$). These results indicate that TFPI-2 expression is silenced by promoter region methylation in primary human esophageal cancer.

Colony formation was inhibited by restoration of TFPI-2 expression

To evaluate the effect of TFPI-2 on clonogenicity of esophageal cancer cells, colony formation assay was employed in KYSE70 cells. Both clone number and size were reduced in TFPI-2 transfected cells compared with empty vector transfected KYSE70 cells (Figure 3). These data suggested that *TFPI-2* is a potential tumor suppressor in esophageal cancer.

The invasion & migration of KYSE70 were inhibited by TFPI-2

TFPI-2 was reported to inhibit glioma cell invasion and migration [12]. To evaluate the effect of TFPI-2 on esophageal cancer invasion, the Transwell assay was employed in TFPI-2 forced expression, empty vector transfected and untreated KYSE70 cells. The invasive cell number of each high powered field in the microscope was 39 ± 8 in the TFPI-2 expressed group, 345 ± 26 in the empty vector group and 373 ± 23 in the untreated cells (Figure 4A). The number of invasive cells is significantly different between the TFPI-2 forced expression group and the empty vector group as well as with the untreated group ($p < 0.0001$). To explore the effect of TFPI-2 on cell migration, the Transwell assay in the absence of ECM coating was performed in KYSE70 cells. The number of migrated cells of each high powered field in the microscope was 33 ± 4 in TFPI-2 forced expression cells, 264 ± 17 in empty vector group, and 272 ± 14 in untreated cells (Figure 4B). The number of migrated cells is significantly different between the TFPI-2 forced expression group and the empty vector group or the untreated group ($p < 0.0001$). Our findings indicate that TFPI-2 suppresses cellular invasion and migration in esophageal cancer cells.

Apoptosis & cell cycle inhibition were induced by TFPI-2

To explore the mechanism of TFPI-2 inhibition of esophageal carcinogenesis, apoptosis was analyzed using the Annexin V-FITC/PI Apoptosis Detection Kit. The ratio of early apoptotic cells was 9.0% in TFPI-2 forced expression cells, 3.4% in the empty vector group, and 0.2% as the basal ratio in KYSE70 cells (Figure 5A). These results indicate that TFPI-2 promotes apoptosis in esophageal cancer cells.

The effect of TFPI-2 on the cell cycle was evaluated by flow cytometry. As shown in Figure 5B, the ratio of G0/1 cells was 32.98%, S phase was 52.42% and G2/M cells was 14.60% in KYSE70 cells transfected with empty vector. The ratio of G0/1 cells was 24.70%, S phase was 50.43% and G2/M cells was 24.87% when TFPI-2 was re-expressed. These results indicate that the ratio of G0/1 and S phase cell is reduced and G2/M phase cell is increased on TFPI-2 re-expression.

To see if TFPI-2 influences esophageal cancer cell proliferation and invasion, the expression of TFPI-2, c-Myc, cyclin D1 and MMP-2 was analyzed by western blot in TFPI-2 expressed and unexpressed KYSE70 cells. As shown in Figure 5C, c-Myc, cyclin D1 and MMP-2 expression were reduced by restoration of TFPI-2 expression. The results suggest that TFPI-2 inhibits esophageal cancer cell proliferation and invasion.

Discussion

TFPI-2 is a Kunitz-type serine protease inhibitor and is secreted in the ECM by different human cells including endothelial cells and fibroblasts [19–21]. TFPI-2 exhibits strong inhibitory activity to a variety of proteinases. Many proteases and their inhibitors are known to be involved in several physiological and pathological processes including angiogenesis, embryo implantation and tumor invasion and migration. Proteolytic degradation of ECM is considered to be an essential step for malignant cells to invade and metastasize to distant tissues. Protease inhibitors are capable of protecting the ECM from degradation and therefore may be used as therapeutic agents for blocking the formation of metastasis.

The ability of TFPI-2 to regulate various proteolytic activities associated with ECM has great relevance to tumor invasion and metastasis [12]. Tissue factor effects on tumor angiogenesis and metastasis required it to be in complex with the catalytically active factor VIIa. TFPI-2 has been reported to inhibit the tissue factor VIIa complex [22,23]. Protease inhibitors (e.g., TFPI-2) and their targets determine tumor characteristics including adhesion, invasion and malignancy. TFPI-2 expression was reported to be gradually

decreased during glioma progression, and anti-invasive effects of TFPI-2 were also found in pancreatic carcinoma [11,24].

Recent studies have shown that *TFPI-2* is frequently methylated in different cancers [13,14,25–28]. However, the role of *TFPI-2* methylation regulation during esophageal carcinogenesis has not been well investigated. To explore the effect of TFPI-2 on esophageal carcinogenesis, we first detected TFPI-2 expression in esophageal cancer cell lines. The results indicate that TFPI-2 loses expression in KYSE70, KYSE150 and KYSE510 cell lines and decreased expression in TE3, YSE2, KYSE30 and KYSE140 cell lines. These results demonstrated that *TFPI-2* may be a tumor suppressor in esophageal cancer. To explore the regulation of TFPI-2, functional methylation studies were performed in esophageal cancer cell lines. The results indicate that TFPI-2 silencing is correlated to promoter region methylation. Regulation of TFPI-2 expression by methylation was further validated by reactivation of *TFPI-2* methylated esophageal cancer cell lines with DAC treatment.

To explore the possibility of *TFPI-2* methylation as an esophageal cancer early detection marker, promoter region methylation was analyzed in normal esophageal mucosa, dysplasia and advanced esophageal cancer tissues. The results show that methylation occurred early and frequently in esophageal cancer, but not in normal mucosa. Moreover, there is a trend of increased methylation with progressive esophageal carcinogenesis (Figure 2). These data indicate *TFPI-2* methylation may be a suitable candidate as an early detection marker and that TFPI-2 may play an important role in esophageal cancer. Further analysis was performed to see the association of *TFPI-2* methylation and clinical factors. As shown in Table 1, *TFPI-2* methylation is significantly related to tumor differentiation (χ^2 test, $p = 0.0323$). Further, the promoter region methylation of *TFPI-2* is inversely related to its expression in paired esophageal cancer and adjacent tissue (χ^2 test, $p = 0.0039$). These results demonstrate that TFPI-2 is regulated by DNA methylation in esophageal primary cancer and that TFPI-2 induces esophageal cancer differentiation.

To further study the function of TFPI-2 in esophageal cancer, colony formation assays were employed in the KYSE70 cell line. As shown in Figure 3, colony number and size were reduced in TFPI-2 expressed cells compared with the empty vector transfected or untreated KYSE70 cells. These data suggest that *TFPI-2* is a potential tumor suppressor in esophageal cancer. The effect of TFPI-2 on invasion and migration was evaluated by the Transwell assay in KYSE70 cells. The number of invasive and migrated cells was significantly reduced in TFPI-2 expressed group compared with the empty vector or untreated groups ($p < 0.0001$) (Figure 4). This hints that TFPI-2 inhibits cellular invasion and migration in esophageal cancer. As shown in Figure 5, TFPI-2 induced apoptosis in esophageal cancer cell lines, and re-expression of TFPI-2 reduced the ratio of G0/1 and S phase cells and increased G2/M phase cells. c-Myc, cyclin D1 and MMP-2 expression were reduced after restoration of TFPI-2 expression. The above results suggest that *TFPI-2* is a tumor suppressor in esophageal cancer.

Conclusion

TFPI-2 is frequently methylated in esophageal cancer and its expression is regulated by DNA methylation. The methylation of *TFPI-2* is increasing during esophageal carcinogenesis indicating that it may serve as an early detection marker in esophageal cancer. *TFPI-2* is a tumor suppressor and may play an important role in esophageal carcinogenesis.

Future perspective

The list of DNA methylation markers for cancer detection, prognosis and predicting therapeutic responses is increasing. *TFPI-2* methylation marker for the early detection of esophageal cancer. Multicenter validation may be necessary for future clinical applications. Epigenetic therapy is becoming an effective approach for different tumor. New strategies are expected to develop epigenetic targeting therapy in reversing a key gene or a group of methylated genes.

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References

Papers of special note have been highlighted as:

■ of interest

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J. Clin.* 2005; 55(2):74–108. [PubMed: 15761078]
2. Adams L, Roth MJ, Abnet CC, et al. Promoter methylation in cytology specimens as an early detection marker for esophageal squamous dysplasia and early esophageal squamous cell carcinoma. *Cancer Prev. Res. (Phila.)*. 2008; 1(5):357–361. [PubMed: 19137073]
3. Toh Y, Oki E, Ohgaki K, et al. Alcohol drinking, cigarette smoking, and the development of squamous cell carcinoma of the esophagus: molecular mechanisms of carcinogenesis. *Int. J. Clin. Oncol.* 2010; 15(2):135–144. [PubMed: 20224883]
4. Lin DC, Du XL, Wang MR. Protein alterations in ESCC and clinical implications: a review. *Dis. Esophagus.* 2009; 22(1):9–20. [PubMed: 18564170]
5. Zhang XM, Guo MZ. The value of epigenetic markers in esophageal cancer. *Front. Med. China.* 2010; 4(4):378–384. [PubMed: 21107750]
6. El-Serag HB, Graham DY, Satia JA, Rabeneck L. Obesity is an independent risk factor for GERD symptoms and erosive esophagitis. *Am. J. Gastroenterol.* 2005; 100(6):1243–1250. [PubMed: 15929752]
7. Campbell NP, Villafor VM. Neoadjuvant treatment of esophageal cancer. *World J. Gastroenterol.* 2010; 16(30):3793–3803. [PubMed: 20698042]
8. Guo M, Ren J, Brock MV, Herman JG, Carraway HE. Promoter methylation of HIN-1 in the progression to esophageal squamous cancer. *Epigenetics.* 2008; 3(6):336–341. [PubMed: 19098448]
9. Kisiel W, Sprecher CA, Foster DC. Evidence that a second human tissue factor pathway inhibitor (TFPI-2) and human placental protein 5 are equivalent. *Blood.* 1994; 84(12):4384–4385. [PubMed: 7994054] ■ First report of *TFPI-2*.
10. Rao CN, Liu YY, Peavey CL, Woodley DT. Novel extracellular matrix-associated serine proteinase inhibitors from human skin fibroblasts. *Arch. Biochem. Biophys.* 1995; 317(1):311–314. [PubMed: 7872799]
11. Tang Z, Geng G, Huang Q, et al. Expression of tissue factor pathway inhibitor 2 in human pancreatic carcinoma and its effect on tumor growth, invasion, and migration *in vitro* and *in vivo*. *J. Surg. Res.* 2011; 167(1):62–69. [PubMed: 20018303]
12. Gessler F, Voss V, Seifert V, Gerlach R, Kogel D. Knockdown of TFPI-2 promotes migration and invasion of glioma cells. *Neurosci. Lett.* 2011; 497(1):49–54. [PubMed: 21530612] ■ Discusses how *TFPI-2*s related to tumor migration and invasion.

13. Nobeyama Y, Okochi-Takada E, Furuta J, et al. Silencing of tissue factor pathway inhibitor-2 gene in malignant melanomas. *Int. J. Cancer*. 2007; 121(2):301–307. [PubMed: 17372906]
14. Wang S, Xiao X, Zhou X, et al. TFPI-2 is a putative tumor suppressor gene frequently inactivated by promoter hyper methylation in nasopharyngeal carcinoma. *BMC Cancer*. 2010; 10:617. [PubMed: 21062455] ■ *TFPI-2s* frequently methylated in nasopharyngeal carcinoma.
15. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA*. 1996; 93(18):9821–9826. [PubMed: 8790415]
16. Jia Y, Yang Y, Liu S, Herman JG, Lu F, Guo M. SOX17 antagonizes WNT/beta-catenin signaling pathway in hepatocellular carcinoma. *Epigenetics*. 2010; 5(8):743–749. [PubMed: 20716954]
17. Griffiths EA, Gore SD. DNA methyltransferase and histone deacetylase inhibitors in the treatment of myelodysplastic syndromes. *Semin. Hematol*. 2008; 45(1):23–30. [PubMed: 18179966]
18. Qin T, Jelinek J, Si J, Shu J, Issa JP. Mechanisms of resistance to 5-aza-2'-deoxycytidine in human cancer cell lines. *Blood*. 2009; 113(3):659–667. [PubMed: 18931345]
19. George J, Gondi CS, Dinh DH, Gujrati M, Rao JS. Restoration of tissue factor pathway inhibitor-2 in a human glioblastoma cell line triggers caspase-mediated pathway and apoptosis. *Clin. Cancer Res*. 2007; 13(12):3507–3517. [PubMed: 17575213] ■ Suggests that *TFPI-2* may induce apoptosis in glioblastoma.
20. Xu C, Deng F, Mao Z, et al. The interaction of the second Kunitz-type domain (KD2) of TFPI-2 with a novel interaction partner, prosaposin, mediates the inhibition of the invasion and migration of human fibrosarcoma cells. *Biochem. J*. 2012; 441(2):665–674. [PubMed: 21943334] ■ Explores the invasion and migration effect of *TFPI-2* on fibrosarcoma.
21. 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(2):583–592. [PubMed: 21224345] ■ Demonstrates that miRNA-616 induced prostate cancer cells proliferation by suppressing *TFPI-2*.
22. Ollivier V, Bentolila S, Chabbat J, et al. Tissue factor-dependent vascular endothelial growth factor production by human fibroblasts in response to activated factor VII. *Blood*. 1998; 91(8):2698–2703. [PubMed: 9531578]
23. Sprecher CA, Kisiel W, Mathewes S, et al. Molecular cloning, expression, and partial characterization of a second human tissue-factor-pathway inhibitor. *Proc. Natl Acad. Sci. USA*. 1994; 91(8):3353–3357. [PubMed: 8159751]
24. Rao CN, Lakka SS, Kin Y, et al. Expression of tissue factor pathway inhibitor 2 inversely correlates during the progression of human gliomas. *Clin. Cancer Res*. 2001; 7(3):570–576. [PubMed: 11297250]
25. Wu D, Xiong L, Wu S, Jiang M, Lian G, Wang M. TFPI-2 methylation predicts poor prognosis in non-small cell lung cancer. *Lung Cancer*. 2011 (Epub ahead of print). ■ Discusses how *TFPI-2* methylation may serve as a lung cancer prognosis marker.
26. 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(5):1129–1138. [PubMed: 17464989]
27. Guo H, Lin Y, Zhang H, et al. Tissue factor pathway inhibitor-2 was repressed by CpG hypermethylation through inhibition of KLF6 binding in highly invasive breast cancer cells. *BMC Mol. Biol*. 2007; 8:110. [PubMed: 18053161]
28. Glockner SC, Dhir M, Yi JM, et al. Methylation of TFPI2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer. *Cancer Res*. 2009; 69(11):4691–4699. [PubMed: 19435926] ■ Suggests TFPI-2 methylation is a colorectal cancer detection marker in stool sample.

Aims

- To explore the epigenetic changes and the function of TFPI-2 in esophageal cancer.

Materials & methods

- *TFPI-2* methylation was examined by methylation-specific PCR in nine esophageal cancer cell lines, nine normal esophageal mucosa, 60 esophageal dysplasia tissues and 106 advanced esophageal cancer tissues.
- Immunohistochemistry was performed to evaluate TFPI-2 expression in primary esophageal cancer and adjacent tissue.
- The effect of TFPI-2 on proliferation, apoptosis, invasion and migration was analyzed by colony formation assay, western blot analysis, transwell assay and flow cytometric analysis.

TFPI-2 methylation may serve as an early detection marker & therapeutic target in esophageal cancer

- *TFPI-2* was frequently methylated in human esophageal cancer. Methylation of *TFPI-2* was in progression during esophageal carcinogenesis.
- *TFPI-2* methylation was significantly related to tumor differentiation ($p = 0.0323$), and TFPI-2 staining was inversely correlated with DNA methylation in esophageal cancer and adjacent tissues ($p = 0.0039$).
- *TFPI-2* methylation was reversible by 5-aza-2'-deoxycytidine in esophageal cancer. It suggests that TFPI-2 is a potential epigenetic therapeutic target.

TFPI-2 inhibition of invasion & migration & induction of apoptosis in esophageal cancer

- Colony formation was inhibited by re-expression of TFPI-2.
- The invasive and migrated cells were reduced in the TFPI-2 re-expressed KYSE70 cell line.
- Re-expression of TFPI-2-induced apoptosis and G2/M check-point arrest.
- Expression of c-Myc, cyclin D1 and MMP-2 was reduced when restoration of TFPI-2 expression in esophageal cancer cell line.

Discussion & conclusion

- *TFPI-2* was frequently methylated in esophageal cancer.
- *TFPI-2* methylation was related to esophageal cancer differentiation.
- The methylation of *TFPI-2* was increasing during esophageal carcinogenesis and may serve as an early detection marker.
- Silencing of TFPI-2 may cause colony formation, invasion, migration and proliferation in esophageal cancer. It is a candidate tumor suppressor for esophageal cancer.

Figure 1. TFPI-2 expression was silenced by DNA methylation in esophageal cancer cell lines
(A) Expression of TFPI-2 was analyzed by semiquantitative RT-PCR in esophageal cancer cell lines (TE3, TE10, YSE2, KYSE30, KYSE70, KYSE140, KYSE150, KYSE450 and KYSE510) before and after DAC treatment. **(B)** MSP results of the *TFPI-2* gene in esophageal cancer cell lines (TE3, TE10, YSE2, KYSE30, KYSE70, KYSE140, KYSE150, KYSE450 and KYSE510). Primer efficiency was verified by a positive control (IVD) and negative control (NL). **(C)** BSSQ of the *TFPI-2* in the promoter region of esophageal cancer cell lines. *TFPI-2* was methylated in KYSE70, partially methylated in KYSE30 and unmethylated in KYSE450 cell lines. The region amplified by MSP is labeled with a double-headed arrow. Filled circles represent methylated CpG sites and open circles denote unmethylated CpG sites.
 -: Before DAC treatment; +: After DAC treatment; BSSQ: Bisulfite sequencing; DAC: 5-aza-2'-deoxycytidine; ddwater: Double distilled water; IVD: *In vitro* methylated DNA; M:

Methylated alleles; MSP: Methylation-specific PCR; NL: Normal blood lymphocyte DNA; TSS: Transcriptional start site; U: Unmethylated alleles.

Figure 2. Loss of TFPI-2 expression was related to promoter region hypermethylation in human primary esophageal cancer

(A) MSP results of *TFPI-2* in normal mucosa, dysplasia and cancer tissues of esophagus. (A1) MSP results of *TFPI-2* in NE. (A2) MSP results of *TFPI-2* in ED. (A3) MSP results of *TFPI-2* in primary EC. (A4) Methylation frequency of *TFPI-2* in normal esophageal epithelia, grade 1 (ED1), grade 2 (ED2), grade 3 (ED3) dysplasia and advanced EC. No methylation was found in NE. Grade 2 and grade 3 dysplasia were merged as one group because of sample size. For advanced EC, stage I, II, III and IV were merged together. (B) Immunohistochemistry analysis of *TFPI-2* in EC and adjacent tissue (×100). (B1) Primary EC, *TFPI-2* was methylated and no positive *TFPI-2* staining was found. (B2) Adjacent tissue of EC, *TFPI-2* was methylated and no positive *TFPI-2* staining was found. (B3) Primary EC, *TFPI-2* was unmethylated and *TFPI-2* staining was found in cytoplasm. (B4) Adjacent tissue of EC, *TFPI-2* was unmethylated and positive staining was found in cytoplasm.

EC: Esophageal cancer; ED: Esophageal dysplasia; IVD: *In vitro* methylated DNA; M: Methylated alleles; NE: Normal esophageal mucosa; NL: Normal blood lymphocyte DNA; U: Unmethylated alleles.

Figure 3. Colony formation was inhibited by TFPI-2 in KYSE70 cells

(A) The size of each clone is different. The size of clones was smaller in the TFPI-2-expressed group compared with the empty vector group in KYSE70 cells. No clone was formed in the absence of vectors with antineomycin after G418 screening. (B) The clone number was smaller in the TFPI-2-expressed group compared with the empty vector group. No clone was formed in the absence of vectors with antineomycin after G418 screening. (C) Average number of tumor clones presented by a bar chart. Each experiment was repeated in triplicate.

Figure 4. The effect of TFPI-2 on cellular invasion and migration

(A) The number of invasive cells was significantly reduced in the TFPI-2-expressed group compared with the empty vector or untreated groups ($p < 0.0001$). (B) The number of migrated cells was significantly reduced in the TFPI-2-expressed group compared with the empty vector or untreated groups ($p < 0.0001$).

Figure 5. The effect of TFPI-2 on apoptosis, cell cycle and proliferation

(A) Apoptosis was evaluated by using the Annexin V-FITC/PI Apoptosis Detection Kit. Three figures represent KYSE70 cells without any treatment, transfected with the empty vector or the TFPI-2-expression vector separately. (B) The cell cycle was analyzed by flow cytometry. The figures and histogram represent cell cycle distribution of KYSE70 cells transfected with the empty vector or the TFPI-2-expression vector separately. (C) The effect of TFPI-2 on cell proliferation was analyzed by western blot analysis in KYSE70 cells. TFPI-2, c-Myc, cyclin D1 and MMP-2 expression were evaluated in untreated and TFPI-2 transfected KYSE70 cells, β-actin was used as control. Dip: Diploid cycle; PI: Propidium iodide.

Table 1
Clinicopathologic characteristics and *TFPI-2* methylation status in human esophageal cancer.

Clinical parameter	n	TFPI-2 methylation status		p-value [†]
		Methylated n = 71 (67%)	Unmethylated n = 35 (33%)	
Age (years)				
<50	13	7 (53.8%)	6 (46.2%)	p = 0.2823
50	93	64 (68.8%)	29 (31.2%)	
Gender				
Male	76	49 (64.5%)	27 (35.5%)	p = 0.3823
Female	30	22 (73.3%)	8 (26.7%)	
Alcohol abuse				
Negative	59	42 (71.2%)	17 (28.8%)	p = 0.3023
Positive	47	29 (61.7%)	18 (38.3%)	
Smoking				
Negative	58	39 (67.2%)	19 (32.8%)	p = 0.9501
Positive	48	32 (66.7%)	16 (33.3%)	
Tumor type				
EAC	8	7 (87.5%)	1 (12.5%)	p = 0.3334
ESCC	97	63 (65.0%)	34 (35.0%)	
Others	1	1 (100%)	0 (0%)	
Tumor size (cm)				
<5	87	59 (67.8%)	28 (32.2%)	p = 0.6957
5	19	12 (63.2%)	7 (36.8%)	
Differentiation				

Clinical parameter	n	TFPL2 methylation status		p-value [†]
		Methylated n = 71 (67%)	Unmethylated n = 35 (33%)	
Poor	40	32 (80.0%)	8 (20.0%)	p = 0.0323 [*]
Moderate	56	35 (62.5%)	21 (37.5%)	
Well	10	4 (40.0%)	6 (60.0%)	
Tumor stage				
I	1	1 (100%)	0 (0%)	p = 0.3312
II	45	28 (62.2%)	17 (37.8%)	
III	55	37 (67.3%)	18 (32.7%)	
IV	5	5 (100%)	0 (0%)	
Metastasis				
Negative	72	46 (63.9%)	26 (36.1%)	p = 0.3246
Positive	34	25 (73.5%)	9 (26.5%)	

[†] p-values are obtained from χ^2 test.

* Statistically significant, $p < 0.05$.

EAC: Esophageal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma.