

Tissue factor pathway inhibitor-2 inhibits the growth and invasion of hepatocellular carcinoma cells and is inactivated in human hepatocellular carcinoma

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Abstract. Human tissue factor pathway inhibitor-2 (TFPI-2) is an extracellular matrix-associated Kunitz-type serine proteinase inhibitor that inhibits the plasmin- and trypsin-mediated activation of matrix metalloproteinases and inhibits tumor progression, invasion and metastasis. Previous studies have shown that TFPI-2 is downregulated in the progression of various tumors. The purpose of this study was to investigate the expression and function of TFPI-2 in hepatocellular carcinoma (HCC). *In situ* hybridization was used to detect human TFPI-2 mRNA and immunohistochemistry was performed to examine the role of TFPI-2 expression in hepatocarcinoma tissues. Cell proliferation was assessed using MTT assay. *In situ* hybridization and immunohistochemical analyses revealed that the expression of TFPI-2 in hepatocarcinoma tissues was markedly lower than that in tumor-adjacent normal hepatic tissues. Restored expression of TFPI-2 in HepG₂ cells inhibits cell proliferation and invasion. Taken together, the results suggest that TFPI-2 has a tumor-suppression action and its inactivation may contribute to HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common primary tumors worldwide (1), and is particularly prevalent in China. It is a major complication of liver cirrhosis. HCC is characterized by a high rate of invasion and metastasis (2-4). Tumor invasion depends on extracellular matrix (ECM)-degrading

proteases, particularly matrix metalloproteinases (MMPs) in the tumor microenvironment (5,6). Particularly in highly aggressive malignant tumors, MMPs are overexpressed in tumor and/or stromal cells and secreted into the ECM (7-9).

Tissue factor pathway inhibitor-2 (TFPI-2) is a 32-kDa serine protease inhibitor comprising three kunitz-type domains homologous to TFPI, also known as placental protein 5 (PP-5) and matrix serine protease inhibitor (MSPI) (10,11). The gene that encodes for TFPI-2 has been mapped to chromosome 7q22 by fluorescence *in situ* hybridization (12). TFPI-2 is abundantly expressed in full-term placenta and is widely expressed in a variety of adult human tissues such as liver, skeletal, muscle, heart, kidney and pancreas (11-13). It is mainly synthesized and secreted into the ECM by a wide variety of cells. Given its pericellular location, TFPI-2 is thought to regulate the plasmin- and trypsin-mediated activation of matrix pro-metalloproteinases and play a significant role in the regulation of ECM degradation, which is an essential step for cell remodeling, as well as tumor cell invasion and metastasis (14).

Little is currently known regarding the role of protease inhibitors, particularly tissue factor pathway inhibitors, in HCC progression. Therefore, in this study, the role of TFPI-2 in HCC is examined.

Materials and methods

Tissue specimens. Human hepatocarcinoma tissues and tumor-adjacent normal hepatic tissues were obtained from HCC patients admitted to Shenzhen People's Hospital. The tissues were stored frozen at -75°C until use.

***In situ* hybridization.** Tumor specimens were fixed in formalin overnight and embedded in paraffin using standard procedures. Series sections (4 µm) were deparaffinized with xylene, rehydrated in a graded series of ethanol and washed in PBS. Human TFPI-2 mRNA was detected using the *in situ* hybridization detection kit (Boster, Wuhan, China) according to the manufacturer's instructions. Briefly, the sections were hybridized in prehybridization buffer supplemented with 0.1 µg/ml digoxigenin-labeled, 1.2-kb antisense TFPI-2 probe overnight

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at 37°C, incubated with biotinylated mouse anti-digoxigenin antibody (1:1000 dilution), and then incubated with biotinylated peroxidase. Staining was developed with DAB. Slides were counterstained with hematoxylin, dehydrated and mounted. The number of cells stained brown (indicating the presense of TFPI-2 mRNA) was assessed by light microscopy. The hybridization probe, replaced with phosphate-buffered saline (PBS) was used as a negative control. Mature placenta tissue, known to express large amounts of TFPI-2, was used as a positive control.

Immunohistochemistry. Tissue sections were prepared in the same manner as above. The expression of TFPI-2 was then determined by incubation with a mouse polyclonal antibody against human TFPI-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), horseradish peroxidase (HRP)-conjugated sheep anti-mouse 1 gG secondary antibodies (Chinagen, Shenzhen, China). Detection was carried out using the non-biotin-labeled detection kit (Zhongshan Goldbridge, Beijing, China) according to the manufacturer's instructions. Staining was developed with DAB, and slides were counterstained with hematoxylin, dehydrated and mounted. The primary antibody, replaced with PBS, was used as a negative control. Mature placenta tissue, known to express large amounts of TFPI-2, was used as a positive control.

Plasmid construct. A 0.7-kb fragment encoding TFPI-2 cDNA was amplified from normal liver tissue with the primers 5'-GCTTTCTCGGACGCCTTGC-3' and 5'-GAATACGACC CCAAGAAATGAGTGA-3'. PCR product was purified and cloned into the *Bam*HI and *Xho*I sites of the pCDNA3.1-expressing vector, donated by Dr Tiyyuan Li (Central Laboratory, Shenzhen People's Hospital, China). The DNA sequence of the recombinant plasmid was confirmed via DNA sequencing.

Cell culture and transfection. Human hepatoma HepG₂ cells were obtained from the Cancer Institute, Chinese Academy of Medical Sciences, and cultured in 6% CO₂ to 94% air and 96% humidity at 37°C in DMEM supplemented with 10% bovine calf serum (Hyclone, Logan, UT, USA), 1.0% glutamine, 100 µg/ml streptomycin and 100 µg/ml penicillin. The recombinant constructs or pCDNA3.1 vector was transfected into HepG₂ cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Selection of transfected cells with 0.8 mg/ml G418 sulfate (Invitrogen) was initiated 48 h after transfection. After a 4-week selection, stable transfectants were expanded and used for the study. The HepG₂ cells were divided into three groups: HepG₂ parental cells (HepG₂-P), HepG₂ cells transfected by pCDNA 3.1 vector (HepG₂-V) and HepG₂ cells transfected by TFPI-2 construct (HepG₂-TFPI-2).

RT-PCR. Total RNA was isolated from HepG₂ cells using TRIzol® reagent (Invitrogen) according to a standard protocol. Using the 2-step (RT-PCR) kit (Takara, Dalian, China), cDNA was synthesized with RNA as the template. PCR amplification of human TFPI-2 and β-actin was performed with Taq Master Mix (Promega, Madison, WI, USA) with synthesized cDNA. The primers were synthesized by Shanghai Biotechnology (China) as follows: TFPI-2 5'-ATAGGATCCACATGGACCCGCTCGC-3' and 5'-GGCCTCGAGAAATTGCTTCTCCGAATTTCC-3',

amplicon 700 bp β-actin 5'-CTGGCACCACACCTTCTAC AATG-3' and 5'-AATGTCACGCACGATTTCCCGC-3'. The PCR conditions were: denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 40 sec for 32 cycles. After electrophoresis of PCR products, the data were analyzed using Image Master Total Laboratory ID software (GE Healthcare, USA). The level of TFPI-2 mRNA was calculated by the ratio of density of TFPI-2 to β-actin.

Western blot analysis. HepG₂-P HepG₂-TFPI-2 and HepG₂-V cells were grown to 80-90% confluence in 6-well plates, after which the medium was replaced with serum-free medium and incubated for 24 h. The cultures were then washed several times with PBS and the ECM was prepared as described by Rao *et al* (15). The ECM protein was supplemented with PMSF (1 mmol/l) to inhibit the proteases. The samples were mixed with equal volume of 2X SDS sample buffer and boiled for 5 min. Equal amounts of protein were resolved on 12% sodium dodecyl sulfate (SDS)-polyacrylamid gels, and then transfected onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated, first with primary antibody at 4°C overnight and then with horseradish peroxidase (HRP)-conjugated sheep anti-mouse 1 gG secondary antibody. After washing, the blots were developed with a super-enhanced chemiluminescence detection kit (Applygen Technologies, Beijing, China).

Cell proliferation assay. Cell proliferation was evaluated by MTT assay (Sigma, St. Louis, MO, USA) according to a procedure described previously (16). In brief, every 24 h, for a total of 7 days, the cells from the three groups were harvested and 200 µl cell suspension was added to each well in 96-well plates. A one-tenth volume of MTT solution (5 mg MTT/ml PBS) was added to each well and incubated for 2-4 h at 37°C until a purple precipitate was visible. The medium was then carefully removed, and precipitates were dissolved in 150 µl DMSO. Growth rate was plotted as the percentage of viable cells in HepG₂-P control (a value arbitrarily set at 100%). Each experiment was repeated at least three times with each treatment given in duplicate or triplicate. Data were presented as an average of the results from individual experiments.

In vitro cell migration and invasion assay. Invasion and migration of the hepatocarcinoma cells *in vitro* was measured by the invasion of cells through Matrigel-coated or -uncoated transwell inserts according to a procedure described previously (17). Briefly, transwell inserts (Corning, Lowell, MA, USA) with 8-µm pore size were coated (for invasion assay) with 50 µg of matrigel matrix (BD Biosciences, San Jose, CA, USA) or uncoated (for the migration assay). Cells suspended in serum-free DMEM medium were seeded into upper chambers (100 µl/well) at a density of 3x10⁵ cells/ml (for the invasion assay) or 1x10⁶ cells/ml (for migration assay). The lower chambers were filled with DMEM and supplemented with 10% FBS. After 24 h of incubation, cells attached to the upper side of the filter were removed, and the filters were fixed and stained with hematoxylin and eosin. At this point, no difference was evident in the total number of cells (proliferation rate) among the groups in the serum-free medium. The number of

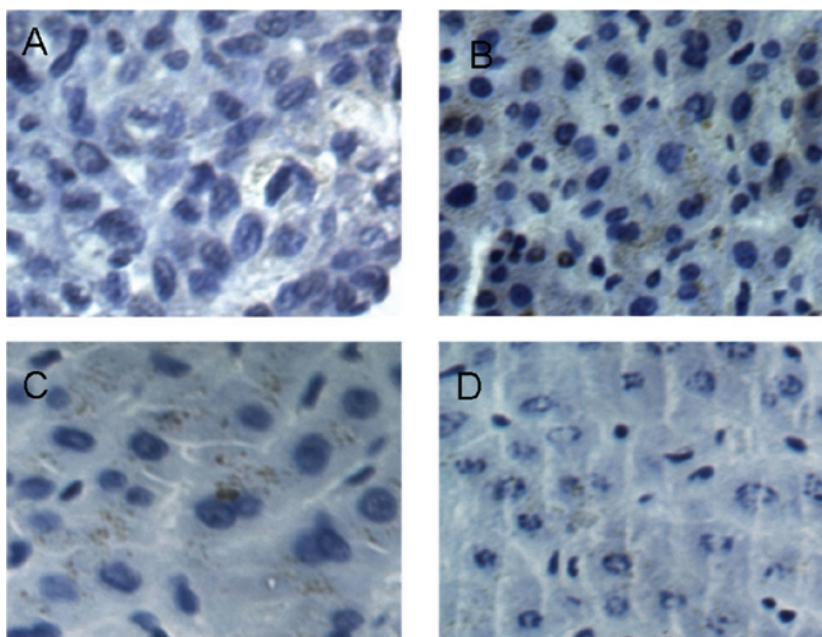


Figure 1. TFPI-2 expression in normal hepatic and hepatocarcinoma tissues. Expression of (A) TFPI-2 mRNA in hepatocarcinoma tissue and (B) tumor-adjacent normal hepatic tissue was examined by *in situ* hybridization with a digoxigenin-labeled TFPI-2 probe. Expression of (C) TFPI-2 protein in hepatocarcinoma tissue and (D) tumor-adjacent normal hepatic tissue was examined by immunohistochemical analyses with TFPI-2 antibody. Magnification, x400.

cells that had migrated to the undersurface of the membrane was counted in five randomly-selected microscopic fields in each sample.

Statistical analysis. Data were presented as the mean \pm SD. Statistical analysis was performed with SPSS statistical software. The Student's two-tailed t-test was used to compare the difference between groups. $P < 0.05$ was considered to be statistically significant.

Results

TFPI-2 expression in normal hepatic and hepatocarcinoma tissues. *In situ* hybridization with TFPI-2 probe detected little or no TFPI-2 mRNA in hepatocarcinoma tissue sections, whereas a high level of TFPI-2 mRNA was detected in tumor-adjacent normal hepatic tissue sections (Fig. 1A and B). The positive and negative controls confirmed the specificity of the hybridization liquid, replaced with PBS and used as a negative control, and the absence of a specific hybridization signal (data not shown).

Further immunohistochemical analysis confirmed that TFPI-2 protein was stained strongly positive in normal hepatic tissues but was weakly stained in hepatocarcinoma tissues (Fig. 1C and D). The TFPI-2 immunostaining scores for normal hepatic tissues and hepatocarcinoma tissues were 46.60 ± 1.80 and 22.54 ± 1.22 , respectively ($P < 0.05$). Taken together, these data indicate that the expression of TFPI-2 was markedly reduced in hepatocarcinoma tissues.

TFPI-2 inhibits HepG₂ cell proliferation in vitro. To determine the functional role of TFPI-2 in HCC, HepG₂ cells were employed as a model. Using RT-PCR, the expression of TFPI-2 mRNA in HepG₂ cells was undetectable (data not

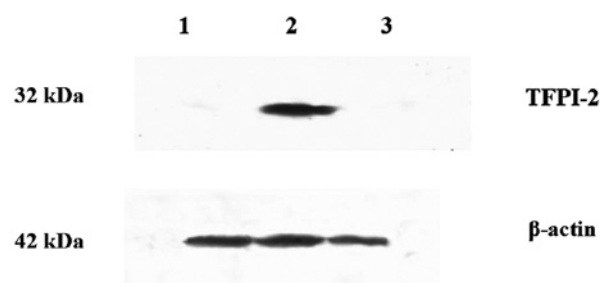


Figure 2. Secretion of TFPI-2 in the conditioned media from HepG₂-TFPI-2 cells. The conditioned media were collected from lanes 1, HepG₂-P cells; 2, HepG₂-TFPI-2 cells and 3, HepG₂-V cells, and analyzed by Western blotting.

shown). Therefore, TFPI-2 was introduced into HepG₂ cells by establishing a HepG₂-TFPI-2 stable cell line. Using Western blotting, a high level of TFPI-2 protein was detected in the conditioned media of the HepG₂-TFPI-2 cells but not in that of HepG₂-V or HepG₂-P cells (Fig. 2). These results showed that TFPI-2 was successfully introduced into HepG₂ cells.

The effect of TFPI-2 expression on the proliferation of HepG₂ cells was then examined. Cell viability was determined by MTT assay for 7 days and cell proliferation was clearly inhibited on the fourth day in HepG₂-TFPI-2 cells but not in the remaining two groups of cells (Fig. 3). These results suggested that TFPI-2 suppressed the growth of hepatocarcinoma cells.

TFPI-2 inhibits HepG₂ cell invasion in vitro. The effect of TFPI-2 expression on the invasion of HepG₂ cells was also examined. Based on the invasion and migration assays, the cells that passed through the membranes were counted (Table I). The results show that the number of cells passing through the membranes was significantly lower in the HepG₂-TFPI-2

Table I. *In vitro* invasion of different groups of HepG2 cells.

Groups	Membranes in matrigel invasion assay					$\chi \pm s$
	1	2	3	4	5	
HepG ₂ -TFPI-2	45	53	39	48	41	49.3 \pm 5.9 ^a
HepG ₂ -V	91	93	80	79	88	86.2 \pm 6.4
HepG ₂ -P	90	86	93	81	89	87.8 \pm 4.5

Groups	Membranes in migration assay					$\chi \pm s$
	1	2	3	4	5	
HepG ₂ -TFPI-2	142	132	139	152	137	140.4 \pm 7.4
HepG ₂ -V	134	148	160	140	147	145.8 \pm 9.8
HepG ₂ -P	150	166	159	142	147	152.8 \pm 9.6

^aP<0.05 vs. HepG₂-V or HepG₂-P.

group than in the remaining two groups (P<0.05), indicating that TFPI-2 suppresses the invasive potential of hepatocarcinoma cells, whereas no significant difference in migration ability was observed in the three groups (Table I).

Discussion

TFPI-2 is a serine proteinase inhibitor, which is frequently downregulated in malignant tumors (18). Previous studies have demonstrated that silencing of TFPI-2 by either histone deacetylation (19) or promoter hypermethylation contributes to its inactivation and tumor progression in a variety of cancers including glioma (18), choriocarcinoma (20), pancreatic carcinoma (17), lung carcinoma (21), breast cancer (22), melanoma (23) and hepatocarcinoma (24). In addition, the aberrant splicing form of TFPI-2 was detected during cancer progression (25), which represents an untranslated form providing another mechanism by which TFPI-2 is downregulated in tumor cells.

In this study, the expression and role of TFPI-2 in HCC were investigated. First, *in situ* hybridization and immunohistochemistry methods were applied to evaluate the expression of TFPI-2 mRNA and protein in hepatocarcinoma and tumor-adjacent normal hepatic tissues. Consistent with previous studies, the results showed that TFPI-2 expression at the mRNA and protein levels was low in hepatocarcinoma tissues compared to adjacent normal hepatic tissues. These results indicated that a decreased expression of TFPI-2 is involved in HCC.

To determine the mechanism by which TFPI-2 loss contributes to HCC, HepG₂ cells were employed as a model. The results demonstrate that reconstitution of TFPI-2 into HepG₂ cells inhibited the proliferation and invasion of HepG₂ cells. Although the details of TFPI-2-mediated growth suppression are unknown, a previous study suggested that TFPI-2 induces apoptosis in glioma cells (26). Further studies are required to examine whether TFPI-2 promotes the apoptosis of HepG₂ cells. In agreement with previous reports asserting that the overexpression of TFPI-2 reduced the invasion of cancer

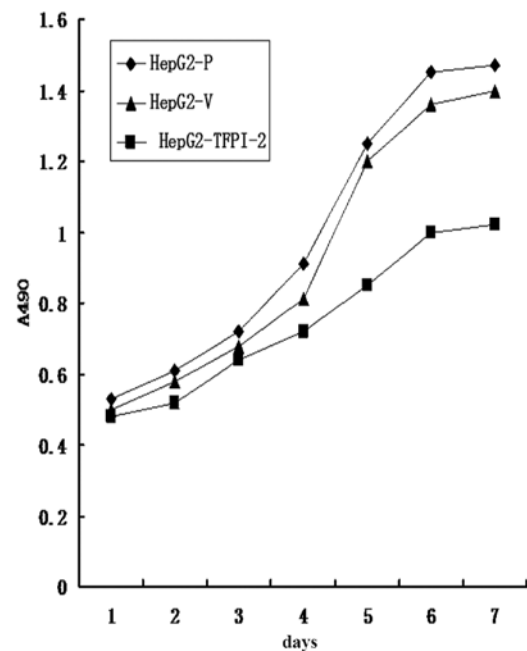


Figure 3. The growth curve of different groups of HepG₂ cells (HepG₂-P, HepG₂-V and HepG₂-TFPI-2).

cell lines derived from melanoma (27), prostate cancer (28), choriocarcinoma (29), glioblastoma (30) or meningiomas (31), our results showed that the restoration of TFPI-2 was associated with a 2-fold decrease in the invasive ability of HepG₂ cells. TFPI-2 is thought to play a pivotal role in the regulation of plasmin-mediated ECM proteolysis during tumor invasion and metastasis (14). TFPI-2 inhibits the release of plasmin and trypsin, preventing activation of pro-matrix metalloproteinase (MMP)-1 and pro-MMP-3, leading to diminished ECM degradation and decreased invasion of HT-1080 fibrosarcoma cell lines (32,33). In addition, TFPI-2 is capable of inhibiting MMP-2 activation in the HT-1080 fibrosarcoma cell line (34) and directly inhibits MMP-1, MMP-13, MMP-2 and MMP-9

in experimental models (35). Thus, we postulate that additional mechanisms responsible for TFPI-2 inhibit HCC invasion and metastasis by modulating the activity of MMPs.

In conclusion, we reported that TFPI-2 expression is lost in HCC. The results of our *in vitro* studies confirm that restoration of TFPI-2 caused decreased proliferative and invasive behaviors of HepG₂ cells. Taken together, these data suggest that inactivation of TFPI-2 contributes to malignant behavior in hepatocarcinoma, suggesting the potential role of TFPI-2 as a tumor suppressor in HCC. However, additional *in vivo* studies may be useful to determine whether restoration of TFPI-2 in hepatocarcinoma cells is a novel therapeutic approach for HCC.

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