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# Tissue Factor Pathway Inhibitor-2 Inhibits the Growth and Invasion of Hepatocellular Carcinoma Cells and is Inactivated in Human Hepatocellular Carcinoma

Yong Xu et al.\*

Pingshan People's Hospital, Shenzhen, Guangdong  
P.R. China

## 1. Introduction

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. Here we aim to investigate the expression and function of TFPI-2 in hepatocellular carcinoma (HCC). *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 HepG<sub>2</sub> cells inhibits cell proliferation and invasion. Taken together, our results suggest that TFPI-2 plays tumor-suppression function and its inactivation may contribute to HCC.

## 2. TFPI-2 expression in normal hepatic and hepatocarcinoma tissues

### 2.1 Tissue specimens

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

### 2.2 In situ hybridization

Tumor specimens were fixed in formalin overnight and embedded in paraffin using standard procedures. Series sections (4μm) were deparaffinized with xylene, rehydrated in a graded series of ethanol, and washed in PBS. Human TFPI-2 mRNA was detected using the

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\* Xiaolin Qin<sup>2</sup>, Jinjing Zhou<sup>2</sup>, Zhiguang Tu<sup>2</sup>, Xiao Bi<sup>3</sup>, Wuxian Li<sup>2</sup>, Xiaoqing Fan<sup>2</sup> and Yi Zhang<sup>3</sup>

<sup>1</sup>Pingshan People's Hospital, Shenzhen, Guangdong, P.R. China

<sup>2</sup>Key Laboratory of Laboratory Medical Diagnostics, Ministry of Education, Chongqing Medical University, Chongqing, P.R. China

<sup>3</sup>The Second Clinical Medical College, Jinan University, Shenzhen People's Hospital, Guangdong, P.R. China

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 ug/ml digoxigenin-labeled, 1.2-kb antisense TFPI-2 probe overnight at 37°C and incubated with biotinylated mouse antidigoxigenin antibody (1:1000 dilution), 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 presence of TFPI-2 mRNA) were assessed by light microscopy. The hybridization probe 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.

### 2.3 Immunohistochemistry

Tissue sections were prepared in the same manner as above. Then the expression of TFPI-2 was determined by incubation with a mouse polyclonal antibody against human TFPI-2 (Santa Cruz, CA), horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG secondary antibodies (Chinagen, Shenzhen, China), and final detection using the non Biotin-labeled

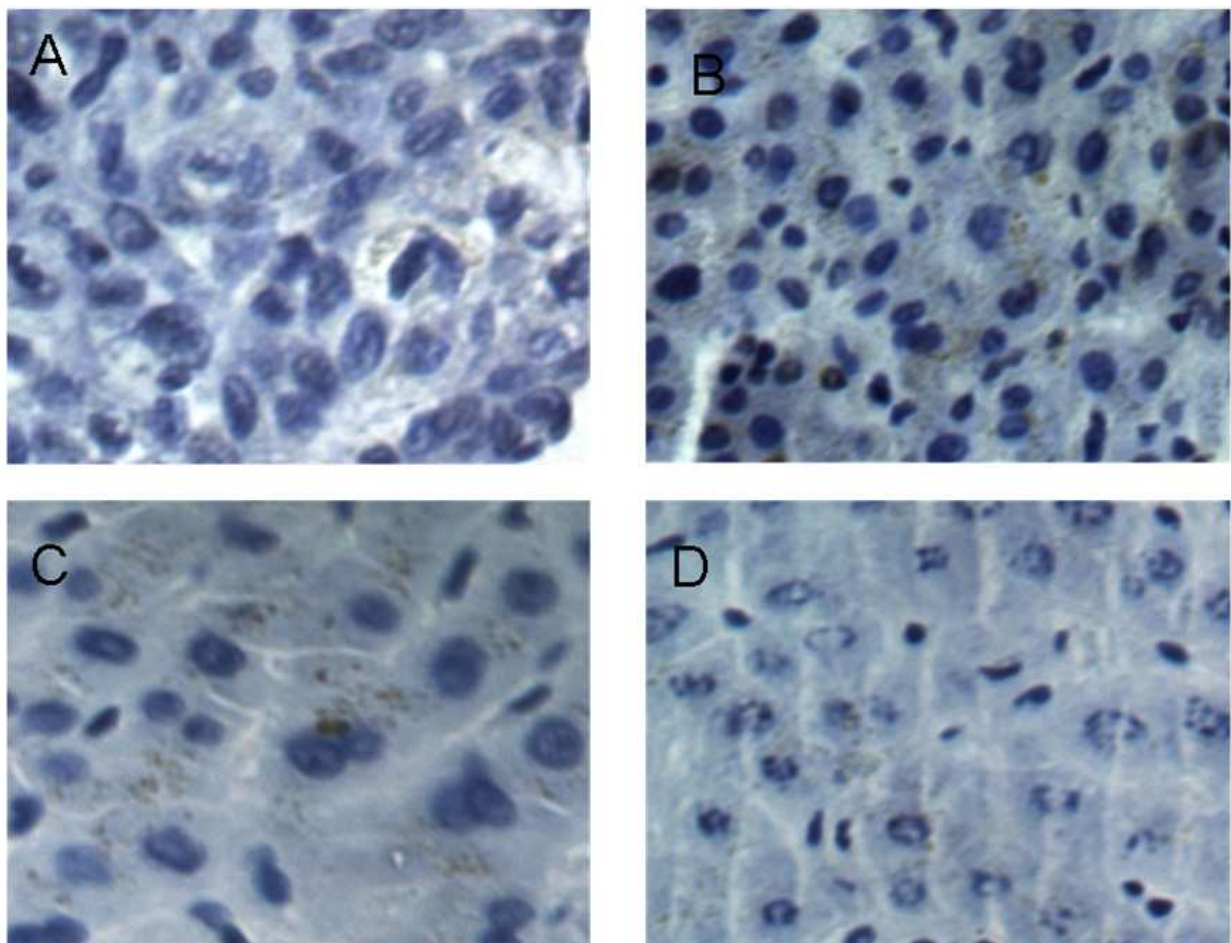


Fig. 1. TFPI-2 expression in normal hepatic and hepatocarcinoma tissues. Expression of (A) TFPI-2 mRNA in hepatocarcinoma tissue and (B) tumor-adjacent normal hepatic tissue was examined by *in situ* hybridization with a digoxigenin-labeled TFPI-2 probe. Expression of (C) TFPI-2 protein in hepatocarcinoma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

Detection Kit (Zhongshan Goldbridge, Beijing, China) according to the manufacturer's instructions. Staining was developed with DAB, 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.

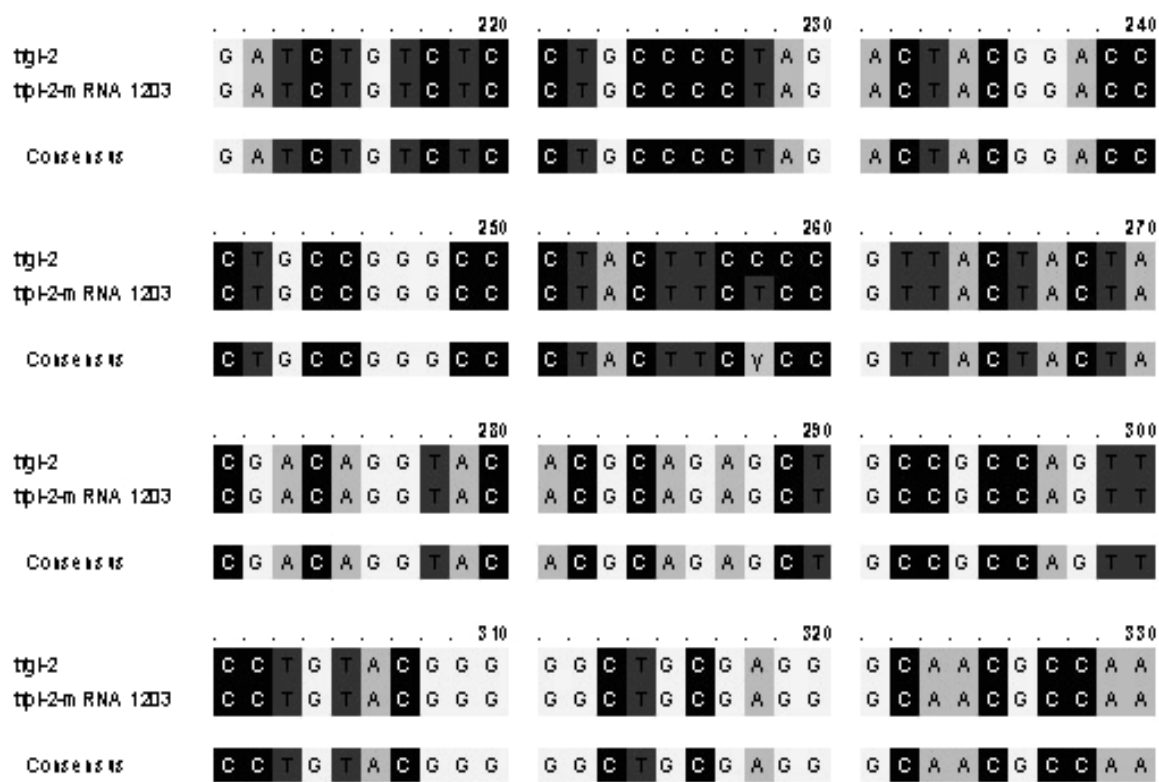
*In situ* hybridization with TFPI-2 probe demonstrated that little or no TFPI-2 mRNA was detected in hepatocarcinoma tissue sections, while a high level of TFPI-2 mRNA was detected in tumor-adjacent normal hepatic tissue sections (Fig. 1A, B). The positive and negative controls confirmed the specificity of hybridization liquid replaced with PBS used as a negative control confirmed 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, D). The TFPI-2 immunostaining scores for normal hepatic tissues and hepatocarcinoma tissues were  $46.60 \pm 1.80$  and  $22.54 \pm 1.22$ , respectively ( $P < 0.05$ ). Taken together, these data indicate that the expression of TFPI-2 was markedly reduced in hepatocarcinoma tissues.

3. TFPI-2 plasmid expression vector construct

3.1 TFPI-2 gene sequencing

The RNA from hepatic tissue of human fetor (Shenzhen People's Hospital) was isolated and full length TFPI-2 cDNA was amplified with RT-PCR kit (TaKaRa). The cloned gene was inserted into plasmid pcDNA2.1 (Chinagen, Shenzhen, China), and sequenced from forward and reverse direction at Shanghai Biotechnology (China), then it was inserted into eukaryotic expression vetor pcDNA3.1, a gift from Dr Tiyuan Li (Central Laboratory, Shenzhen People's Hospital) verified by enzyme digestion and sequencing.



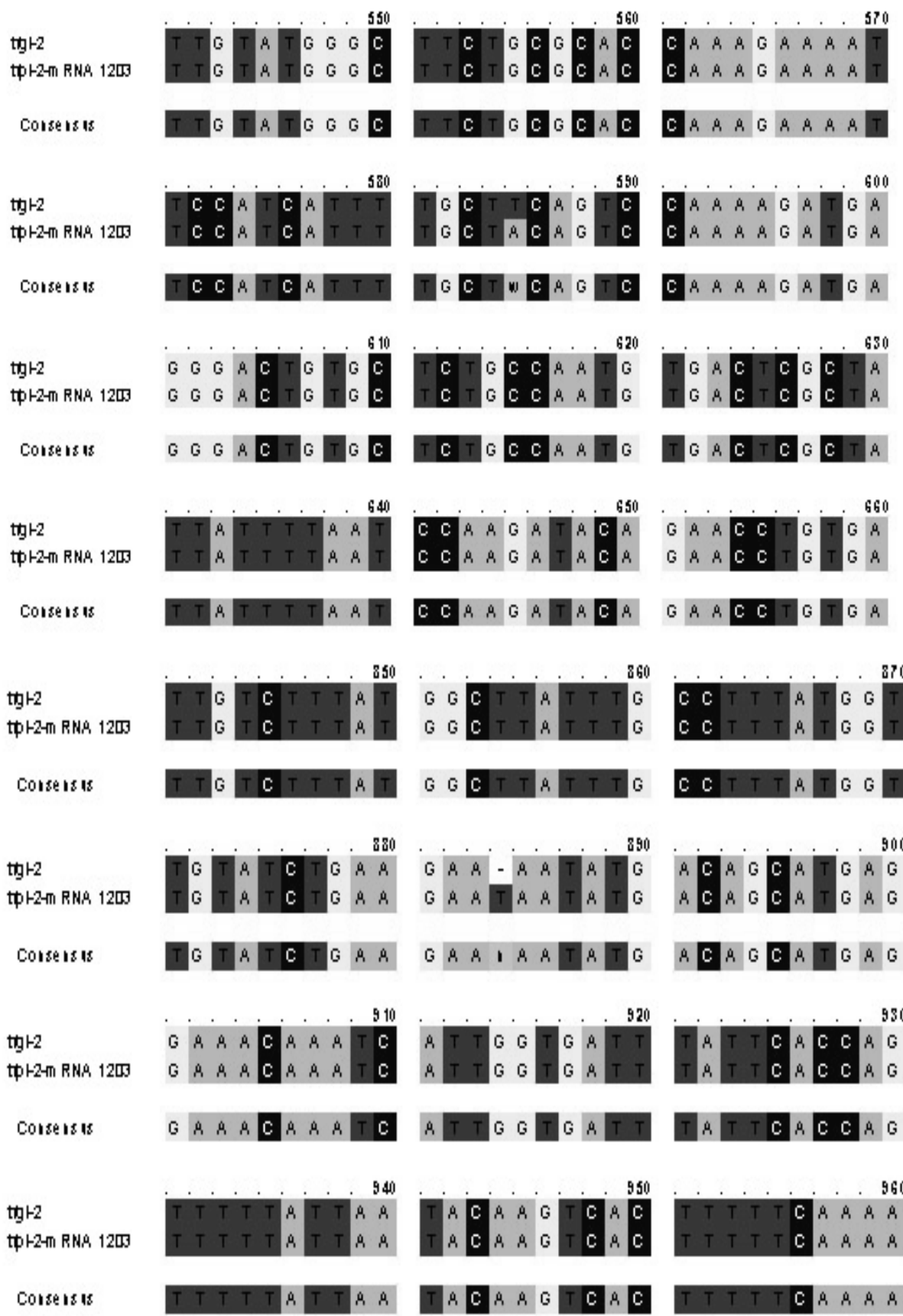


Fig. 2. Homology analysis of TFPI-2 gene



### 3.2 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'-GAATACGACCCCAAGAAATGAGTGA-3'. PCR product was purified and cloned into the BamHI and XhoI sites of the pCDNA3.1 expressing vector. The DNA sequence of the recombinant plasmid was confirmed via DNA sequencing.

The Chinese TFPI-2 gene is 1222bp. Sequencing results showed that the cloned Chinese TFPI-2 gene has three bases different (258, 585 and 884 bp) with that registered in Genbank (Fig. 2). Our sequencing results has been transmitted and accepted by Genbank, accession number is TFPI AY691946. TFPI-2 gene was inserted to eukaryotic expression vector pCDNA3.1 successfully. The result of nucleotide sequencing confirmed that the recombinant vector pCDNA3.1-TFPI-2 was constructed accurately.

## 4. Construct HepG<sub>2</sub>-TFPI-2 stable cell line

### 4.1 Cell culture and transfection

To explore the functional role of TFPI-2 in HCC, we employed HepG<sub>2</sub> cells as a model. Based on RT-PCR, we found that the expression of TFPI-2 mRNA in HepG<sub>2</sub> cells was undetected (data not shown), therefore we introduced TFPI-2 into HepG<sub>2</sub> cells by establishing HepG<sub>2</sub>-TFPI-2 stable cell line.

Human hepatoma HepG<sub>2</sub> cells were obtained from Cancer Institute, Chinese Academy of Medical Sciences, and cultured in 6% CO<sub>2</sub> to 94% air and 96% humidity at 37°C in DMEM supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 1.0% glutamine, 100 ug/ml streptomycin, 100 ug/ml penicillin. The recombinant constructs or pCDNA3.1 vector was transfected into HepG<sub>2</sub> cells using Lipofectamine 2000 transfection reagent (Invitrogen) 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 HepG<sub>2</sub> cells were divided into three groups: HepG<sub>2</sub> parental cells (HepG<sub>2</sub>-P), HepG<sub>2</sub> cells transfected by pCDNA3.1 vector (HepG<sub>2</sub>-V) and HepG<sub>2</sub> cells transfected by TFPI-2 construct (HepG<sub>2</sub>-TFPI-2).

### 4.2 RT-PCR

Total RNA was isolated from HepG<sub>2</sub> cells using TRIZOL reagent (Invitrogen) following a standard protocol. Using the 2-step RT-PCR kit (TaKaRa), cDNA was synthesized with RNA as the template. PCR amplification of human TFPI-2 and  $\beta$ -actin was performed with Taq Master Mix (Promega, Madison, WI, USA) with synthesized cDNA. The primer were synthesized by Shanghai Biotechnology (China) as follows: TFPI-2 5'-

ATAGGATCCACATGGACCCGCTCGC-3'

and 5'-GGCCTCGAGAAATTGCTTCTTCCGAATTTCC-3', amplicon 700 bp.  $\beta$ -actin 5'-CTGGCACCACCTTCTACAATG-3' and 5'-AATGTCACGCACGATTTCCTCCG-3'. The PCR condition were: denaturing 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 by Image Master Total Laboratory ID software. The level of TFPI-2 mRNA was calculated by the ratio of density of TFPI-2 to  $\beta$ -actin.

### 4.3 Western blot

HepG<sub>2</sub>-P HepG<sub>2</sub>-TFPI-2 and HepG<sub>2</sub>-V cells were grown to 80-90% confluence in six-well plates, after which the medium was replaced with serum-free medium and incubated for 24 h. Then the cultures were washed several times with PBS and the ECM was prepared as described by Rao *et al* (15). The ECM protein were supplemented with PMSF (1 mmol/L) to inhibit the proteases. The samples was mixed with equal volume of 2×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 (Millopore). 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 IgG secondary antibody. After washing, the blots were developed with a super-Enhanced Chemiluminescence Detection Kit (Applygen Technologies, Beijing, China).

By Western blot we found that a high level of TFPI-2 protein was detected in conditioned media of HepG<sub>2</sub>-TFPI-2 cells but not in that of HepG<sub>2</sub>-V or the HepG<sub>2</sub>-P cells (Fig. 3). These results proved that we successfully introduced TFPI-2 into HepG2 cells.



Fig. 3. Secretion of TFPI-2 in the conditioned media from HepG<sub>2</sub>-TFPI-2 cells. The conditioned media were collected from lanes 1, HepG<sub>2</sub>-P cells; 2, HepG<sub>2</sub>-TFPI-2 cells and 3, HepG<sub>2</sub>-V cells, and analyzed by Western blotting.

### 5. Cell proliferation assay

Cell proliferation was evaluated by MTT assay (sigma) 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 ul of 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 ul DMSO. Growth rate was plotted as the percentage of viable cells in HepG<sub>2</sub>-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.

We examined the effect of TFPI-2 expression on the proliferation of HepG2 cells. The viability of cells was determined by MTT assay for 7 days and cell proliferation was obviously inhibited on the fourth day in HepG<sub>2</sub>-TFPI-2 cells but not in the other two groups of cells (Fig. 4). These results suggested that TFPI-2 could suppress the growth of hepatocarcinoma cells.

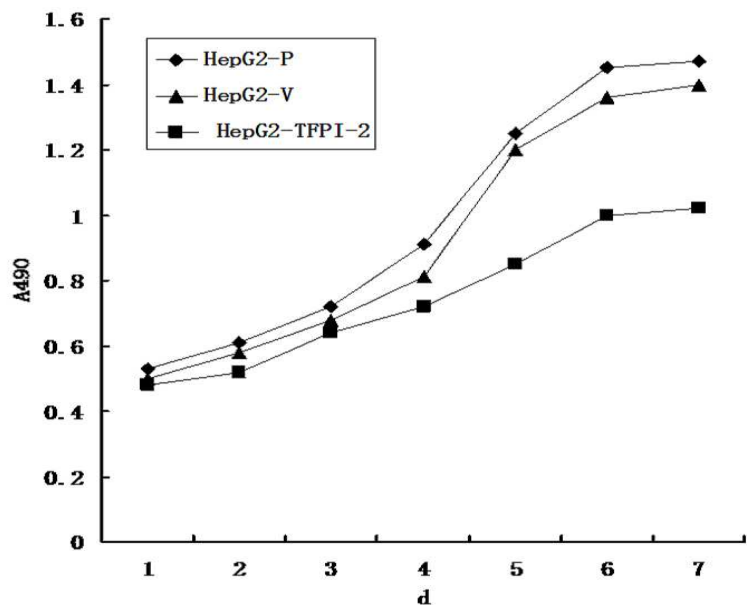


Fig. 4. The growth curve of different groups of HepG<sub>2</sub> cells (HepG<sub>2</sub>-P, HepG<sub>2</sub>-V and HepG<sub>2</sub>-TFPI-2).

6. *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) with 8-um pore size were coated (for invasion assay) or uncoated (for migration assay) with 50 ug of Matrigel matrix (BD Biosciences). Cells suspended in serum-free DMEM medium were seeded into upper chambers (100 ul/well) at a density of 1×10<sup>6</sup> cells/ml (for migration assay) or 3×10<sup>5</sup> cells/ml (for invasion assay). The lower chambers were filled with DMEM 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, there was no difference in the total number of cells (proliferation rate) among the groups in the serum-free medium. The number of cells that had migrated to the undersurface of the membrane was counted in five randomly-selected microscopic fields in each sample.

Groups	Membranes in <b>matrigel invasion assay</b>					$\bar{x} \pm s$
	1	2	3	4	5	
HepG <sub>2</sub> -TFPI-2	45	53	39	48	41	49.3 ± 5.9 *
HepG <sub>2</sub> -V	91	93	80	79	88	86.2 ± 6.4
HepG <sub>2</sub> -P	90	86	93	81	89	87.8 ± 4.5

Groups	Membranes in <b>migration assay</b>					$\bar{x} \pm s$
	1	2	3	4	5	
HepG <sub>2</sub> -TFPI-2	142	132	139	152	137	140.4 ± 7.4
HepG <sub>2</sub> -V	134	148	160	140	147	145.8 ± 9.8
HepG <sub>2</sub> -P	150	166	159	142	147	152.8 ± 9.6

\* p< 0.05 vs HepG2-V or HepG2-P

Table 1. In vitro invasion of different groups of HepG2 cells



We went further to examine the effect of TFPI-2 expression on the invasion of HepG<sub>2</sub> cells. Based on invasion and migration assays, we counted the cells that passed through the membranes (Table 1). The results show that the number of cells passing through the membranes was significantly lower in the HepG<sub>2</sub>-TFPI-2 group than the other two groups ( $P<0.05$ ), indicating that TFPI-2 suppresses the invasive potential of hepatocarcinoma cells. While no significant difference in migration ability was observed in the three groups (Table 1).

## 7. Statistical analysis

All data were presented as mean $\pm$ SD. Statistical analysis was performed with SPSS statistical software. The Student two-tailed  $t$  test was used to compare the difference between groups,  $p<0.05$  was considered to be statistically significant.

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 several 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, we investigated the expression and function of TFPI-2 in HCC. We first applied the *in situ* hybridization and immunohistochemistry methods to evaluate the expression of TFPI-2 mRNA and protein in hepatocarcinoma tissues and tumor-adjacent normal hepatic tissues. Consistent with previous studies, our results showed that TFPI-2 expression at both 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 implicated in HCC.

To find the mechanism by which TFPI-2 loss contributes to HCC, we employed HepG<sub>2</sub> cells as a model. Our results demonstrate that reconstitution of TFPI-2 into HepG<sub>2</sub> cells could inhibit the proliferation and invasion of HepG<sub>2</sub> cells. Although the details for TFPI-2-mediated growth suppression are unknown, a previous study suggested that TFPI-2 induces apoptosis in glioma cells (26). Further studies are necessary to examine whether TFPI-2 promotes apoptosis of HepG<sub>2</sub> cells. In agreement with previous reports that overexpression of TFPI-2 reduced the invasion of cancer cell lines derived from melanoma (27), prostate cancer (28), choriocarcinoma (29), glioblastoma (30) or meningiomas (31), our results showed that restoration of TFPI-2 was associated with a twofold decrease in invasive ability of HepG<sub>2</sub> cells. In fact, 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- or trypsin-dependent activation of pro-matrix metalloproteinase (MMP)-1 and pro-MMP-3, which leads to diminished ECM degradation and decreased invasion of HT-1080 fibrosarcoma cell lines (32, 33). In addition, TFPI-2 can inhibit MMP-2 activation in HT-1080 cells (34) and inhibit MMP-1, MMP-13, MMP-2 and MMP-9 in experimental models (35). Thus we assume that TFPI-2 inhibits HCC invasion and metastasis through modulating the activity of MMPs.

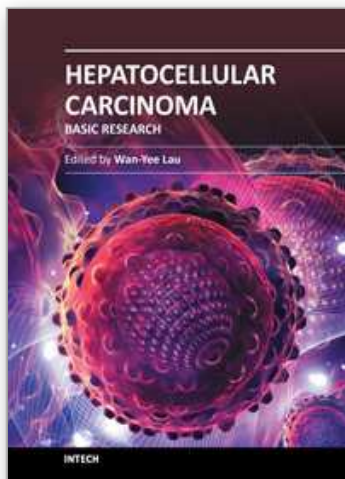
In summary, we reported that TFPI-2 expression is lost in HCC. The results of our *in vitro* studies confirm that restoration of TFPI-2 caused decreased proliferative and invasive behaviors of HepG<sub>2</sub> cells. Taken together, these data suggest that inactivation of TFPI-2 may contribute to the malignant behavior in hepatocarcinoma. Additional *in vivo* studies will

help determine whether restoration of TFPI-2 in hepatocarcinoma cells may represent a novel therapeutic approach for HCC.

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## **Hepatocellular Carcinoma - Basic Research**

Edited by Dr. Joseph W.Y. Lau

ISBN 978-953-51-0023-2

Hard cover, 402 pages

**Publisher** InTech

**Published online** 10, February, 2012

**Published in print edition** February, 2012

Hepatocellular Carcinoma represents a leading cause of cancer death and a major health problem in developing countries where hepatitis B infection is prevalent. It has also become increasingly important with the increase in hepatitis C infection in developed countries. Knowledge of hepatocellular carcinoma has progressed rapidly. This book is a compendium of papers written by experts to present the most up-to-date knowledge on hepatocellular carcinoma. This book deals mainly with the basic research aspect of hepatocellular carcinoma. The book is divided into three sections: (I) Biomarkers / Therapeutic Target; (II) Carcinogenesis / Invasion / Metastasis; and (III) Detection / Prevention / Prevalence. There are 18 chapters in this book. This book is an important contribution to the basic research of hepatocellular carcinoma. The intended readers of this book are scientists and clinicians who are interested in research on hepatocellular carcinoma. Epidemiologists, pathologists, hospital administrators and drug manufacturers will also find this book useful.

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Yong Xu, Xiaolin Qin, Jinjing Zhou, Zhiguang Tu, Xiao Bi, Wuxian Li, Xiaoqing Fan and Yi Zhang (2012). Tissue Factor Pathway Inhibitor-2 Inhibits the Growth and Invasion of Hepatocellular Carcinoma Cells and is Inactivated in Human Hepatocellular Carcinoma, *Hepatocellular Carcinoma - Basic Research*, Dr. Joseph W.Y. Lau (Ed.), ISBN: 978-953-51-0023-2, InTech, Available from: <http://www.intechopen.com/books/hepatocellular-carcinoma-basic-research/tissue-factor-pathway-inhibitor-2-inhibits-the-growth-and-invasion-of-hepatocellular-carcinoma-cells>

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Phone: +86-21-62489820  
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