The Role of Tissue Factor Pathway Inhibitor-2 in Cancer Biology

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ABSTRACT

Tissue factor pathway inhibitor-2 (TFPI-2), a member of the Kunitz-type serine proteinase inhibitor family, is a structural homologue of tissue factor pathway inhibitor (TFPI). The expression of TFPI-2 in tumors is inversely related to an increasing degree of malignancy, which may suggest a role for TFPI-2 in the maintenance of tumor stability and inhibition of the growth of neoplasms. TFPI-2 inhibits the tissue factor/factor VIIa (TF/VIIa) complex and a wide variety of serine proteinases including plasmin, plasma kallikrein, factor XIa, trypsin, and chymotrypsin. Aberrant methylation of TFPI-2 promoter cytosine-phosphorothioate-guanine (CpG) islands in human cancers and cancer cell lines was widely documented to be responsible for diminished expression of mRNA encoding TFPI-2 and decreased or inhibited synthesis of TFPI-2 protein during cancer progression. Furthermore, an aberrantly spliced variant of TFPI-2 mRNA (designated as TFPI-2) was detected, which represents an untranslated form of TFPI-2. The levels of as TFPI-2 were very low or undetectable in normal cells but markedly upregulated in neoplastic tissue. TFPI-2 functions in the maintenance of the stability of the tumor environment and inhibits invasiveness and growth of neoplasms, as well as metastases formation. TFPI-2 has also been shown to induce apoptosis and inhibit angiogenesis, which may contribute significantly to tumor growth inhibition. Restoration of TFPI-2 expression in tumor tissue inhibits invasion, tumor growth, and metastasis, which creates a novel possibility of cancer patient treatment. However, more information is still needed to define the precise role of TFPI-2 in human tumor biology.

KEYWORDS: Coagulation inhibitors, TFPI-2, cancer

STRUCTURE AND FUNCTION OF TFPI-2

The TFPI-2 molecule consists of three tandemly arranged Kunitz-type proteinase inhibitor domains, a negatively charged amino terminal region, and a positively charged carboxy terminal region. The first Kunitz domain with arginine in its P1 reactive site is responsible for TFPI-2 inhibitory activity. Recently, other residues flanking the P1 residue, particularly at P6 and P5, were documented to influence the inhibitory activity and specificity of human TFPI-2. In 1994, Kisiel and co-workers, on the basis of available data on molecular cloning, structure, and function, revealed

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the identity of TFPI-2 with previously described placent- 
cental protein 5 (PP5).6 Three types of TFPI-2 mole- 
cules (33, 31, and 27 kDa), resulting from differential 
glycosylation, were identified in the extracellular matrix 
of human dermal fibroblasts and human umbilical vein 
endothelial cells (HUVECs).7–9

Despite the structural similarity of TFPI-2 and 
TFPI, TFPI-2 serves as a weak inhibitor of tissue factor/ 
factor VIIa (TF/VIIa) complex, in contrast with TFPI.1–3 
The dominant activity of TFPI-2 is directed at the 
inhibition of a wide variety of serine proteinases 
including plasmin,3,6,10,11 plasma kallikrein,3 trypsin 
inhibition of a wide variety of serine proteinases 
and is abundantly secreted (60 to 90%) into the extrac- 
cellular matrix.16 Furthermore, expression of the 
gene encoding TFPI-2 was detected in human ciliary 
epithelium,17 mature placent, heart, liver, kidneys, and 
skeletal muscles.12 TFPI-2 mRNA was also demon- 
strated in syncytiotrophoblasts,18 whereas the product 
of the gene was found both in syncytiotrophoblast and 
cytotrophoblast.19 TFPI-2 protein was also immunohis- 
tochemically detected in seminal vesicles,20 colon, breast, 
pancreas, stomach, larynx, kidney, endometrium, and 
brain tissue.21 The plasma concentration of TFPI-2 in 
men and nonpregnant women is very low (0.43 to 0.49 
ng/mL).19 Moreover, TFPI-2 is present in seminal 
plasma,22 preovulatory follicular fluid,23 and in the 
mucus of the uterus.19

**LOCALIZATION OF TFPI-2 UNDER NORMAL CONDITIONS**

TFPI-2 is synthesized in endothelial cells (ECs) of 
different blood vessels (venous, arterial, and capillaries) 
but predominantly by the ECs of small blood vessels16 
and is abundantly secreted (60 to 90%) into the extrac- 
cellular matrix (ECM).16 Furthermore, expression of the 
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**TFPI-2 PRESENCE IN NEOPLASTIC TISSUES**

Immunohistochemical studies have provided suggestive 
evidence that the expression of TFPI-2 decreases with an 
increasing degree of malignancy (in the case of breast, 
gastric, colon, pancreatic, renal, laryngeal, and endome- 
trial cancer and glial neoplasms).24 The highest levels of 
TFPI-2 mRNA and protein were detected in normal 
brain, with lesser amounts in low-grade gliomas and 
anaplastic astrocytom. In glioblastomas, TFPI-2 was 
undetected.24 An inverse correlation between TFPI-2 
distribution and the degree of malignancy was also 
observed in choriocarcinoma18 and fibrosarcoma cell 
lines.25 In addition, in about one third of non–small 
cell lung cancers (NSCLCs), TFPI-2 gene expression is 
decreased 4- to 120-fold compared with normal lung 
tissue.26,27 Neither pancreatic cancer cell lines nor pri- 
mary pancreatic ductal neoplasms revealed expression 
of TFPI-2 mRNA.28 Interestingly, high expression of 
TFPI-2 was observed exclusively in lobular carcinoma 
of the breast, which is known to be associated with a 
much more favorable prognosis than ductal invasive 
breast carcinoma.21 Moreover, as much as 90% of 
hepatocellular carcinomas were characterized by under- 
expression of TFPI-2.29 Thus, there is ample evidence 
that TFPI-2 production is reduced or absent during 
tumor progression. However, little is known concerning 
the molecular underpinnings of this phenomenon.

**GENETIC AND EPIGENETIC BASIS OF TFPI-2 EXPRESSION IN CANCER**

As mentioned above, the gene for the TFPI-2 is localized 
in chromosome 7,13 which is frequently associated with 
genetic changes in different cancers (e.g., head and neck 
carcinomas,30 gastric,18 colon,31 prostate,31 vesicular,32 
ovarian18 and testicular cancer,13 malignant melanoma, 
and glial neoplasms32). Several highly aggressive cancers 
delete the locus for the TFPI-2 gene in chromosome 7q 
region, which results in a complete lack of TFPI-2 
protein expression in these cells.33–35 In human chorio- 
carcinoma cell line, the minimal TFPI-2 promoter was 
demonstrated to be located between –166 and –111 from 
the translation start site, and nuclear factor-1 (NF-1), 
nuclear factor-kappa B (NF-κB), and early growth re- 
sponse gene-1 (egr-1)/specificity protein 1 (Sp1) binding 
sites were crucial for TFPI-2 inducible expression.15 The 
promoter region –313 to +1 is critical for minimal and 
inducible promoter activity of human TFPI-2 in glioma 
cells.36 This region harbors sites for several transcription 
factors, including SP1, activating enhancer-binding pro- 
ein-1 (AP-1), NF-κB and NF-κB-like site, and lym- 
hphoid transcription factor-1 (Lyf-1).37 Mutations at 
either of two AP-1 sites (–310 to –300 and –163 to 
154), as well as either of two SP1 sites (–192 to 183 and – 
135 to 128), lead to reduced TFPI-2 activity.37 Promoter 
polyorphism of various genes is frequently responsible 
for regulation of functions of particular proteins.36 How- 
ever, none of 90 NSCLC patients exhibited genetic 
variations in the TFPI-2 promoter region.26

Of particular interest is epigenetic inactivation of 
TFPI-2 synthesis. aberrant methylation of TFPI-2 
promoter cytosine-phosphorothioate-guanine (CpG) 
islands in human cancers and cancer cell lines was widely 
documented to be responsible for diminished mRNA 
ecoding TFPI-2 and decreased or abolished synthesis
of TFPI-2 protein during cancer progression.\textsuperscript{27–30,38–41} The finding was associated with increased cancer growth, invasion, and dissemination.\textsuperscript{27–29,38–41} Such a mechanism of TFPI-2 gene silencing was demonstrated in melanoma cell lines (HMV-I, MeWo, and WM-115), as well as in 30\% of metastatic tumors, but not in primary melanoma surgical specimens.\textsuperscript{38} One third of NSCLC patients, particularly at stage III and IV of the disease, exhibited methylation of TFPI-2 gene promoter in cancer cells.\textsuperscript{27} Approximately one half of NSCLC cases with silenced TFPI-2 gene were lymph nodes positive.\textsuperscript{27} TFPI-2 methylation was observed in all cases of esophageal adenocarcinomas examined.\textsuperscript{39} Aberrant methylation of TFPI-2 was also revealed in as many as 73\% of pancreatic cancer xenografts and primary pancreatic adenocarcinomas.\textsuperscript{28} The finding was more frequently detected in older patients.\textsuperscript{28} Interestingly, TFPI-2 methylation in endoscopically aspirated pure pancreatic juice was observed in 60\% of pancreatic cancer patients in contrast with 20\% in patients with intraductal neoplasms or 5\% in individuals with chronic pancreatitis.\textsuperscript{41} The incidence of the aberrant methylation of TFPI-2 in the pure pancreatic juice reached 100\% when liver metastases were present and was as high as 90\% in patients who presented at stage IV of the disease.\textsuperscript{41} Promoter methylation was also documented to be responsible for TFPI-2 gene silencing in SNB19 glioblastoma cell line.\textsuperscript{40} The finding was observed in 80\% of hepatocellular carcinoma cell lines and in 50\% of human hepatocellular carcinomas.\textsuperscript{29}

Although methylation of the TFPI-2 gene promoter leads to its silencing, it does not seem to provide the sole explanation for this phenomenon.\textsuperscript{42} Rao et al\textsuperscript{42} raised the hypothesis that one or more components of pathways regulating TFPI-2 expression had also undergone methylation-associated silencing in cancer cells. In this context, recent observations on the extracellular signal-regulated kinase family (ERK)/mitogen-activated protein kinase (MAPK) pathway mediation of the TFPI-2 promoter activity is of particular interest.\textsuperscript{43} It has also been reported that TFPI-2 expression is downregulated in cells harboring activated ras oncogenes.\textsuperscript{25} The Ras/Raf/mitogen-activity protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling cascade regulates cell proliferation and differentiation.\textsuperscript{44} Activation of components of the latter pathway, specifically oncogenic Ras and constitutively activated ERKs, are frequent findings in various human malignancies.\textsuperscript{45–47}

A novel mechanism, by which tumor cells circumvent the effects of TFPI-2, was recently discovered.\textsuperscript{48} Specifically, an aberrantly spliced variant of TFPI-2 mRNA derived from TFPI-2 pre-mRNA splicing at exon/intron boundaries, as well as at new sites within exons and introns (designated asTFPI-2), was detected.\textsuperscript{48} This form of TFPI-2 mRNA is untranslated.\textsuperscript{48} The levels of asTFPI-2 were very low or negligible in normal cells but markedly upregulated in neoplastic tissue and in several cancer cell lines.\textsuperscript{48} Based on the latter observation, it is conceivable that quantification of asTFPI-2 mRNA levels could be a marker of tumor progression.

Another facet of TFPI-2 regulation derives from its cleavage by other tumor environment components. Recently, an extracellular metalloproteinase, ADAMTS1, was shown to modify the binding properties of TFPI-2 that alter the extracellular location of the protein and disrupt its function.\textsuperscript{49} It is particularly noteworthy that ADAMTS1 is expressed in human malignant tumors and plays a significant role in structural remodeling facilitating cancer invasion processes.\textsuperscript{50}

TFPI-2 gene is becoming increasingly recognized as a tumor suppressor gene. Marked downregulation of TFPI-2 expression is associated with increasing malignancy.\textsuperscript{21,24} However, this observation raises an important question regarding its biological relevance in aggressive tumors.

THE ROLE OF TFPI-2 IN MALIGNANCY

TFPI-2, an ECM-derived inhibitor, may function in the maintenance of the stability of the tumor environment and inhibit the growth of neoplasms. It has been shown to suppress invasion of choriocarcinoma tumor cells both in vitro and in vivo.\textsuperscript{51} Similarly, TFPI-2 decreases invasion of human lung cancer A549cell line,\textsuperscript{52} glioblastoma SNB19 cell line,\textsuperscript{24,53} fibrosarcoma HT-1080 cell line,\textsuperscript{54} prostate cancer LNCaP cell line,\textsuperscript{55} and amelanotic melanoma C-32 cell line.\textsuperscript{56} The invasive potential of cancer derives from proteolytic activity driven by tumor cells, which results in ECM and basement membrane degradation.\textsuperscript{57} In fact, TFPI-2 is thought to play a pivotal role in the regulation of plasin-mediated ECM proteolysis. Downregulation of TFPI-2 protein concentration enhances cancer cell ability to degrade the ECM because TFPI-2 is a potent inhibitor of plasin regardless of whether the enzyme is associated with the tumor cells or ECM.\textsuperscript{3,58} TFPI-2 exerts inhibitory effect on plasin- or trypsin-dependent activation of pro-matrix metalloproteinase (MMP)-1 and pro-MMP-3, which subsequently leads to diminished ECM degradation and decreased invasion of HT-1080 fibrosarcoma cell lines.\textsuperscript{54,59} In addition, it is conceivable that TFPI-2 may inhibit the plasin-mediated release of proangiogenic molecules from the ECM components. Moreover, TFPI-2 has been demonstrated to inhibit MMP-2 formation in HT-1080 fibrosarcoma cell line\textsuperscript{35} and to directly inhibit MMP-1, MMP-13, MMP-2, and MMP-9 in experimental models.\textsuperscript{60} However, recently performed studies revealed that native TFPI-2 failed to form complexes with MMP-2, MMP-9, and MMP-1.\textsuperscript{61} TFPI-2 also failed to inhibit the proteolytic activity of MMP-1 toward triple-helical collagen.\textsuperscript{61} Experimental
studies focused on the effect of restoration of TFPI-2 revealed inhibition of invasion and tumor growth of several cancer lines.\textsuperscript{28,62,63}

A unique characteristics of TFPI-2 were observed in human hepatocellular carcinoma cell line.\textsuperscript{64} The serine protease inhibitor was demonstrated to promote invasion, an effect that was suggested to be a result of TFPI-2 binding to TF-VIIa complex on cancer cells.\textsuperscript{64}

It is not obvious if this phenomenon represents the mechanism by which TFPI-2 exerts its activity in vivo because the results of another study revealed that hypermethylation of its gene promoter was detected in as many as 50% of human hepatocellular carcinomas, and ectopic overexpression of TFPI-2 markedly reduced the proliferation and invasiveness of hepatocellular carcinoma cells.\textsuperscript{29} Significantly higher amounts of TFPI-2 in human glioblastoma cell line (U-251) activates both intrinsic and extrinsic caspase-

Restoration of TFPI-2 in human glioblastoma cell line (U-251) activates both intrinsic and extrinsic caspase-mediated, proapoptotic signaling pathways and induces apoptosis.\textsuperscript{66}

It is thoroughly documented that activation of blood coagulation contributes to local progression of cancer and facilitates metastatic spread as well.\textsuperscript{67–84} Tissue factor plays a key role in the initiation of coagulation activation in malignancy.\textsuperscript{62,82} The presence of TF was observed in loco in many tumors and resulted in increased invasiveness and higher rate of metastases formation.\textsuperscript{67–84} The essential role in TF/VIIa inactivation is played by TFPI.\textsuperscript{1,2} Another inhibitor of TF-dependent pathway of blood coagulation is TFPI-2. It may inhibit tumor growth via interfering with TF activity. However, data on the influence of TFPI-2 on cancer biology by such a mechanism are scanty and will require additional studies. Nonetheless, it seems that there exists a regulatory self-restricting mechanism that, to some extent, counteracts TF influence on cancer progression. Namely, TF activity leads to thrombin generation, which upregulates TFPI-2 synthesis.\textsuperscript{85} In this way, thrombin may contribute to suppression of the extrinsic pathway of blood coagulation and subsequently facilitate the maintenance of ECM and may attenuate TF-mediated impact on cancer progression.

The process of angiogenesis is a rate-limiting step in cancer progression.\textsuperscript{85} Several studies highlight the role of TFPI-2 in the regulation of new blood vessel formation. In this regard, TFPI-2 was shown to inhibit angiogenesis in experimental models.\textsuperscript{86,87} The inhibitor is synthesized by ECs, particularly small blood vessels, and secreted abundantly into the ECM.\textsuperscript{16} It may facilitate the maintenance of integrity of ECM and basement membrane of small blood vessels, thus preventing initiation of angiogenesis. Moreover, TFPI-2 plays a role in EC adhesion as anti-TFPI-2 IgG treatment of ECs leads to their detachment from the ECM.\textsuperscript{16} Of interest, TFPI-2 in ECs is upregulated by the essential proangiogenic growth factor vascular endothelial growth factor (VEGF), which suppress proliferation of ECs.\textsuperscript{88} This may represent the mechanism for negative-feedback regulation of VEGF activity.

Several highly aggressive neoplasms (melanoma as well as breast, lung, prostate, and ovarian cancer) develop a vessel-like network formed by cancer cells themselves; the process known as vasculogenic mimicry.\textsuperscript{89–95} The structures play a role in sustained blood supply of the nascent tumor, and their formation was correlated with tumor progression.\textsuperscript{89–95} In experimental melanoma, TFPI-2 was demonstrated to strongly contribute to vasculogenic mimicry plasticity.\textsuperscript{96}

The presence of TFPI-2 was demonstrated in tumor-infiltrating macrophages in gastric and renal carcinomas.\textsuperscript{21} Cancer cells are able to synthesize and release several different cytokines that exert multidirectional influences on normal host cells\textsuperscript{97} and inflammatory mediators, such as tumor necrosis factor-α (TNF-α), endotoxin, and phorbol esters, which stimulate synthesis and secretion of TFPI-2 by HUVECs.\textsuperscript{9} The precise role of TFPI-2 localized in tumor-associated macrophages in cancer biology is still unknown.

On the basis of gene profiling and protein detection, it is tempting to speculate that TFPI-2 expression is attenuated during cancer progression, which facilitates invasion, tumor growth, angiogenesis, as well as metastases formation. Restoration of TFPI-2 in the tumor tissue inhibits the above-mentioned processes suggesting a novel therapeutic target for the treatment of cancer patients. However, additional information on the precise role of TFPI-2 in the tumor biology of particular types of human cancer is needed to design therapeutic options based on TFPI-2 structure and function.

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