ORIGINAL ARTICLE

TFPI-2 inhibits the invasion and metastasis of bladder cancer cells

TFPI-2 inhibite l’invasion et les métastases des cellules cancéreuses de la vessie

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Summary

Introduction. — Bladder cancer metastasis seriously affects the prognosis of patients, but its molecular mechanism is unclear. This study sought to explore the roles of tissue factor pathway inhibitor-2 (TFPI-2) gene overexpression in the infiltration and metastasis of bladder cancer.

Materials. — Firstly, real-time PCR and immunohistochemistry were used to compare the mRNA and protein expression levels, respectively, of TFPI-2 and matrix metalloproteinase-1 (MMP-1) in adjacent non-tumoral tissues, muscle-invasive bladder cancer (MIBC) tissues, and non-muscle-invasive bladder cancer (NMIBC) tissues. BIU-87-TFPI-2 cells that stably expressed TFPI-2 were generated by transfection with pcDNA3.1-TFPI-2. Real-time PCR and western blotting were performed to determine the mRNA and protein expression levels, respectively, of TFPI-2 and MMP-1 in BIU-87-TFPI-2 cells. The invasion and migration abilities of BIU-87-TFPI-2 cells were investigated using the Transwell chamber method.

Results. — TFPI-2 was found to be significantly downregulated in bladder cancer tissue. The expression of MMP-1 was increased with the progression of bladder cancer. BIU-87 cells that overexpressed TFPI-2 were successfully generated by transfection with pcDNA3.1-TFPI-2. TFPI-2 overexpression in BIU-87 cells significantly inhibited cancer cell invasion and metastasis. Furthermore, the mRNA and protein expression levels of MMP-1 were significantly reduced in TFPI-2-overexpressing cells.

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Introduction

Bladder cancer has a high incidence and is one of the most common genitourinary tumours among men in China. Globally, the incidence of bladder cancer ranks tenth among all cancers, with 549,000 new cases and 200,000 deaths in 2018. The incidence of bladder cancer in men is 4 times that in women [1]. It has a high recurrence rate, and most patients eventually relapse, or the tumour metastasises; malignancy of the tumour increases after recurrence. Its occurrence and development might be related to the abnormal expression of a variety of cancer-related genes.

Tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type protease inhibitor that can inhibit the activity of a variety of proteases. It is widely expressed in normal human tissues, such as the heart, pancreas, liver, kidney, and skeletal muscle [2]. Previous studies have found that TFPI-2 is closely related to the invasion, metastasis, and prognosis of tumours, such as breast cancer [3], lung cancer [4], liver cancer [5], and glioblastoma [6]. It has been found that TFPI-2 expression decreases as the bladder cancer grade progresses [7]. However, the roles of TFPI-2 enhancement in bladder cancer have not yet been studied. This study aimed to investigate whether TFPI-2 overexpression in human bladder cancer cells could inhibit the biological characteristics of malignant tumours.

Materials and methods

Data and methods

Tissue samples and cell lines

This study included 100 patients diagnosed with bladder cancer who underwent surgery at the Department of Urology of the Affiliated Hospital of Putian University from August 2014 to July 2019. All specimens were collected
from cancer tissues and adjacent non-tumoral tissues (postoperative pathology verified). All of the specimens were stored at −80 °C for later use. The human bladder cancer cell line BIU-87 was purchased from China Centre for Type Culture Collection (CCTCC, Wuhan, China). BIU-87 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum at 37 °C and under 5% CO₂. Cells were digested with 0.25% EDTA-trypsin and passaged after 2-3 days of culture. Cells in the logarithmic growth phase were used for the experiments.

Main reagents
The EliVision plus reagent kit was purchased from Fuzhou Maixin Biotechnology Co., Ltd. TRIzol was purchased from Invitrogen (15596-026) (USA). A first-strand cDNA synthesis kit was purchased from Thermo Fisher (K1622) (USA). Real-time PCR Master Mix (SYBR Green) was purchased from TOYOBO (Japan), and the primers were synthesised by Nanjing Springen Co. Ltd. The western blot assay kit was purchased from KeyGen Biotechnology (KGP1201; Jiangsu, China). The TFPI-2 monoclonal antibody was purchased from R&D (USA). The M1-1 monoclonal antibody was purchased from Santa Cruz Biotechnology (USA). The pcDNA3.1-TFPI-2 eukaryotic expression vector was constructed by KeyGen Biotechnology, Jiangsu. Transwell chambers were purchased from Corning Incorporated (USA).

Methods
**TFPI-2 gene expression analysis**
GEPIA (http://geopia.cancer-pku.cn/) was used to analyse and verify the expression of TFPI-2 between normal bladder tissues and bladder cancer tissues. All of the data were generated from the Cancer Genome Atlas (TCGA) Research Network's BLCA dataset.

**Immunohistochemistry**
Conventional paraffin sections (3 consecutive sections) were collected from non-muscle-invasive bladder cancer (NMIBC) tissues, muscle-invasive bladder cancer (MIBC) tissues, and adjacent non-tumoral tissues. Immunohistochemical staining was performed according to the instructions of the EliVision plus Immunohistochemistry kit (Fuzhou). For semi-quantitative evaluation of protein expression levels in tissue, the HSCORE immunoreactivity scoring system was used. The HSCORE was calculated using the following formula: HSCORE = \( \sum \text{Pi}(+1) \), where i is the staining intensity of cells, and Pi is the percentage of cells at each level of intensity [8].

**Real-time PCR**
Total RNA was extracted using the TRIzol method. Total cDNA synthesis and PCR amplification were performed according to the manufacturers' instructions. The cycle thresholds (ct values) of TFPI-2 and GAPDH were used for the calculations of the group internal normalisation value \( \Delta \text{ct} \) (ct value of tissue-the corresponding ct value of internal reference); the \( \Delta \text{ct} \) values calculated through the subtraction of the control group \( \Delta \text{ct} \) from the experimental group \( \Delta \text{ct} \) and were then subjected to the formula \( 2^{-\Delta \text{ct}} \). The data were analysed using the \( 2^{-\Delta \text{ct}} \) method.

**Cell transfection**
Cells were inoculated into 6-well plates and cultured for 24h. Cells were rinsed twice with serum-free RPMI-1640 medium and RPMI-1640 medium containing 10% fetal bovine serum for transfection. The recombinant plasmid pcDNA3.1-TFPI-2 and empty vector pcDNA3.1 were transfected into BIU-87 cells using Lipofectamine in 6-well plates; a non-transfected group was used as the control. The experiment was divided into 3 groups:
- transfected with pcDNA3.1-TFPI-2 (BIU-87-TFPI-2 group);
- transfected with empty vector (BIU-87-V group);
- non-transfected group (BIU-87-P group).

**Western blot detection of TFPI-2 and MMP-1 protein expression before and after transfection**
The extracellular matrix (ECM) proteins were extracted from the 3 groups. The protein concentrations were determined, and the proteins were separated by SDS-PAGE. After separation, the proteins were transferred to PVDF membranes under constant voltage. Subsequently, the membranes were blocked in TBST buffer with 5% fat-free milk at room temperature for 2h and were then incubated with the TFPI-2 polyclonal antibody (diluted at 1:300 using TBST) at 4 °C in a refrigerator overnight. Next, the membranes were washed with TBST 5 times and then incubated with HRP-labelled anti-mouse secondary antibody for 1.5h. Bands were observed in a darkroom. GAPDH served as the internal control.

**Transwell cell invasion and migration experiments in vitro**
**In vitro cell invasion assay**
Matrigel stored at −20 °C was dissolved in 4 °C cell culture medium, mixed well, and placed on a PVDF microporous membrane. After incubation at 37 °C for 120 min, the Matrigel was polymerised to form a gel. Cells in the logarithmic growth phase (BIU-87-TFPI-2 group, BIU-87-V group, and BIU-87-P group) were collected, digested and diluted to obtain \( 10^5 \) cells/mL. One hundred-microlitre cell suspensions from the above 3 groups were added to the upper chamber of the micropore, and 500 µL of medium containing 20% FBS were added to the lower chamber. Transwell chambers were placed in a 37 °C, 5% CO₂ incubator for 24h. The Matrigel and cells in the upper chamber were wiped off with a cotton swab, and then the Transwell was removed. Samples were inverted and air-dried, and 500 µL of 0.1% crystal violet were added. The chamber was placed in the solution, and the membrane was immersed in the dye at 37 °C. After 30min, samples were removed and washed with PBS. Three fields of view were imaged (magnification ×200), and the cells were counted.

**In vitro cell migration assay**
Cells in the logarithmic growth phase (BIU-87-TFPI-2 group, BIU-87-V group, and BIU-87-P group) were digested and diluted to prepare a \( 10^5 \) cells/mL cell suspension. One hundred-microlitre cell suspensions from the above 3 groups were added to the upper chamber of the micropore, and 500 µL of medium containing 20% FBS were added to the lower chamber. The culture, staining, and counting methods were the same as those for the invasion experiments.
The number of transmembrane cells in each group was calculated as an index to evaluate the invasiveness and migration ability of the tumour cells.

**Statistical methods**

The data are expressed as the mean ± standard deviation (\( \bar{x} \pm s \)). SPSS statistical software, version 17.0, was used for the analysis. One-way analysis of variance (ANOVA) was used for the multisample comparisons, and t-tests were used for the two-sample comparisons; differences were considered statistically significant at \( P < 0.05 \).

**Results**

The expression level of TFPI-2 is decreased with the progression of bladder cancer

The GEPIA online tool was used to compare the expression of TFPI-2 in bladder cancer tissues versus normal bladder tissues, finding that TFPI-2 expression in cancer tissues is lower than that in normal bladder tissues (Fig. 1A). The real-time PCR and immunohistochemistry results (Fig. 1B–D) validated that TFPI-2 expression in NMIBC tissues and MIBC tissues was significantly lower than that in adjacent non-tumoral tissues; the difference was statistically significant (\( P < 0.05 \)). TFPI-2 expression in the MIBC tissues was significantly lower than that in the NMIBC tissues; the difference was statistically significant (\( P < 0.05 \)). Above all, these results indicated that TFPI-2 expression is decreased with the progression of bladder cancer, consistent with the results of a previous study [7].

**Correlation between TFPI-2 expression and bladder cancer clinicopathological parameters**

There were 100 patients with bladder cancer included in the current study. The patients comprised of 70 men and 30 women whose ages ranged from 35 to 78 years old, with a mean age of 52.5 ± 7.4 years. There were 80 cases of NMIBC tissues and 20 cases of MIBC tissues. The correlations between the mRNA expression levels of TFPI-2 and selected clinicopathological variables are summarised in Table 1. T-tests were used to analyse categorical variables, and differences were considered statistically significant at \( P < 0.05 \). The expression of TFPI-2 was significantly decreased in tumours with higher grades and stages (\( P < 0.05 \)). TFPI-2 expression was not altered in tumours with different sizes (\( P > 0.05 \)). TFPI-2 expression decreased in tumours with lymph node metastasis (\( P < 0.01 \)). The expression of TFPI-2 was significantly decreased in relapsed tumours (\( P < 0.01 \)) (Table 1).

**The expression level of MMP1 is increased with the progression of bladder cancer**

The real-time PCR and immunohistochemistry results (Fig. 1B–D) showed that MMP1 expression in NMIBC tissues and MIBC tissues was significantly higher than that in adjacent non-tumoral tissues; the difference was statistically significant (\( P < 0.05 \)). MMP1 expression in the MIBC tissues was significantly higher than that in the NMIBC tissues; the difference was statistically significant (\( P < 0.05 \)).

**Enhanced expression of TFPI-2 in bladder cancer cells**

Since the expression of TFPI-2 is decreased with the progression of bladder cancer, we sought to enhance TFPI-2 expression in the bladder cancer cell line BIU-87 (ref) by transfection with the recombinant plasmid pcDNA3.1-TFPI-2. qRT-PCR revealed an increase in the TFPI-2 mRNA levels after transfection (Fig. 2A). Western blot analysis showed that the TFPI-2 expression (molecular weight, 27 kDa) increased after transfection (Fig. 2B and C). These results indicated that the BIU-87-TFPI-2 cells that we generated could stably express enhanced TFPI-2.

**TFPI-2 overexpression in bladder cancer cells inhibited cell invasion and migration in vitro**

The number of cells in the BIU-87-TFPI-2 group that migrated across the transmembrane was significantly smaller than those in the BIU-87-V and BIU-87-P groups (Fig. 3A–G, \( P < 0.05 \)), suggesting that TFPI-2 inhibited the invasion and migration capabilities of bladder cancer cells in vitro.

**TFPI-2 overexpression in bladder cancer cells inhibited MMP-1 expression**

To understand how TFPI-2 overexpression exerts its inhibitory effects in bladder cancer cells, we examined the expression of MMP-1, one of the matrix-metalloproteinases that is closely correlated with the invasive ability of glioma, in BIU-87-TFPI-2 cells. Both the mRNA and protein expression of MMP-1 significantly decreased after overexpression of TFPI-2 (Fig. 2A–C). Considered together, our results suggested that TFPI-2 overexpression could inhibit bladder cancer cell invasion and migration, which are likely mediated by the inhibition of MMP-1 activity.

**Discussion**

The main treatment methods for bladder cancer include surgery and radio-chemotherapy interventions [9]. Although there are many treatment methods for bladder cancer, the recurrence rate of bladder cancer is high, and the degree of malignancy is often higher after relapse. Therefore, how to reduce the postoperative recurrence and metastasis rates of bladder cancer has attracted substantial research interest.

TFPI-2 is a matrix-associated serine protease inhibitor, formerly known as placental protein 5, which belongs to the Kunitz-type serine protease inhibitor family [10]. Research findings [11,12] have indicated that TFPI-2 inhibits the activity of multiple enzymes, such as MMP-1, plasminogen, chymotrypsin, and trypsin, and inhibits the degradation of extracellular matrix proteins, playing an important role in tumour cell invasion and metastasis. The expression of TFPI-2 in some malignant tumours is significantly reduced or even absent. Here, we showed that TFPI-2 was highly
Figure 1. A. TFPI-2 mRNA expression in primary human bladder cancer tissue samples compared to normal bladder tissue samples by the online tool GEPIA. B. TFPI-2 and MMP1 mRNA expression in adjacent non-tumoral tissue, NMIBC tissue, and MIBC tissue. C,D. TFPI-2 and MMP1 protein expression in adjacent non-tumoral tissue, NMIBC, and MIBC ($\times$ 200 times). *P < 0.05.

Table 1  Clinicopathologic characteristics and mRNA expression ($2^{-\Delta \Delta CT}$) of TFPI-2 in bladder cancer.

<table>
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<th>TFPI-2 mRNA expression</th>
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<td></td>
<td></td>
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expressed in adjacent non-tumoral tissues but was significantly decreased in NMIBC tissues and especially in MIBC tissues, suggesting that the reduction or loss of TFPI-2 expression might be related to the invasion of bladder cancer. In this study, the TFPI-2 gene was successfully transfected into BIU-87 bladder cancer cells by liposome-mediated transfection. After transfection, the invasion and migratory capabilities were inhibited, consistent with the
finding that TFPI-2 gene expression reduces the invasive ability of hepatoma cells [13]. To the best of our knowledge, our study is the first to reveal the role of TFPI-2 gene overexpression in the inhibition of the invasion and migratory capabilities of bladder cancer cells in vitro.

As a member of the MMP family, MMP-1 is a serine protease that can degrade the basement membrane and extracellular matrix [14,15], while degradation of the extracellular matrix is an important step in tumour cell invasion and metastasis, and the secretion and activity of MMPs are increased in many malignant tumours [16]. This study showed that MMP1 expression in NMIBC tissues and MIBC tissues was significantly higher than that in adjacent non-tumour tissues. The expression level of MMP1 was increased with the progression of bladder cancer. MMP-1 expression was significantly reduced in bladder cancer cells transfected with TFPI-2, indicating that TFPI-2-mediated inhibition of cancer cell invasion and migration might occur through the inhibition of MMP-1 activity.

Conclusion

Bladder cancer is a common urinary tract cancer with a high incidence and recurrence rate. We showed here that exogenous TFPI-2 overexpression could inhibit the invasion and migration of BIU-87 bladder cancer cells in vitro. Our study therefore provides unambiguous evidence to demonstrate that the TFPI-2 gene is a target worth exploring during the development of alternative drugs for the treatment of bladder cancer.

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Disclosure of interest

The authors declare that they have no competing interest.

References