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ORIGINAL ARTICLE

TFPI-2 expression is decreased in bladder cancer and is related to apoptosis

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Summary

Purpose: *This study aimed to study the role of TFPI-2 in bladder cancer and its relation with apoptosis.*

Methods: *Immunohistochemical (IHC) staining of TFPI-2 and TUNEL were applied. By semiquantitative analysis of the IHC data, we compared the TFPI-2 expression with clinicopathological parameters of 24 bladder cancer samples. TUNEL assay was used to study the apoptotic level of bladder cancer cells. Also, quantitative PCR and Western blot were used to confirm IHC results.*

Results: *The expression of TFPI-2 decreased with progression of bladder cancer grade ($p<0.001$) and tumor stage ($p<0.001$). Also, TFPI-2 expression was not significantly decreased in smaller and single tumors ($p=0.536$ and $p=0.378$, respectively). Increased TFPI-2 expression was significantly correlated with increased apoptosis ($p<0.001$). Lower TFPI-2 was also correlated with lower Ki67 index but not with TP53 positivity ($p=0.003$ and $p=0.195$, respectively). Expression of TFPI-2 detected by IHC was consistent with that detected by Western blotting and PCR.*

Conclusion: *TFPI-2 expression was decreased in bladder cancer. TFPI-2 expression was decreased with progression in tumor grade and stage and was correlated to decreased apoptosis. Our findings indicated that TFPI-2 could be a marker of bladder cancer and enhancement of TFPI-2 could combat bladder cancer.*

Key words: *apoptosis, bladder cancer, TFPI-2*

Introduction

Bladder cancer, also referred to as urothelial carcinoma of the urinary bladder, is one of the most common malignancies of the urinary system and is characterized by multicentricity, high recurrence rates and potent invasiveness [1]. The variety of current therapies for bladder tumors e.g. surgical resection, chemotherapy, immunotherapy and radiotherapy, cannot decrease satisfactorily the recurrence rates which can be as high as 80%, of which 16–25% are of higher grade and 10% are invasive [2]. Therefore, identification of novel tumor markers could contribute to early detection and novel disease treatments.

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) [3]. The expression of TFPI-2 in tumors is inversely related to an increasing grade of malignancy, which may suggest that TFPI-2 can inhibit the growth of neoplasms. TFPI-2 is an inhibitor of MMPs (matrix metalloproteinases), which can promote invasiveness of tumors through matrix degradation [4]. The protein levels of TFPI-2 were very low or undetectable in many tumor cells compared to normal cells. TFPI-2 contributes in the maintenance of

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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 [5].

In the current study, we have investigated the expression of TFPI-2 in bladder cancer, as its role in bladder cancer has yet to be demonstrated. Herein we have shown that decreased TFPI-2 expression was associated with progression of the disease. Our results supported the role of a novel tumor marker for TFPI-2.

Methods

Patient data collection

Twenty-four fresh tissue samples from patients diagnosed with bladder cancer were included in the current study. Tissue samples were taken either via transurethral resections (TURBT) or radical cystoprostatectomies, all performed at Huashan Hospital between Jan 2011 and Dec 2013. All patients undergoing TURBT were primarily diagnosed and patients undergoing cystectomies were previously treated with TURBT for at least one time. Each fresh sample was subject to different assays, namely paraffinization, protein extraction, and RNA extraction. Ethical approval was acquired from Huashan Hospital Institutional Review Board (HIRB). All sections were re-stained with hematoxylin-eosin and reassessed by 2 independent pathologists. All samples were graded and staged according to the WHO/ISUP (2004) and UICCTNM (2002), respectively [6,7]. Clinicopathological data was collected and analysed.

Immunohistochemical staining (IHC)

A standard IHC protocol using the EnVision system was applied in our previous reports [8-15]. Briefly, all formalin-fixed and paraffin-embedded samples were cut into 4-μm thick sections and mounted on polylysin-coated slices. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide. Sections for TFPI-2 staining (Santa Cruz, CA) were treated with boiling at pH 6.0 for 20 min. Non-specific antigen sites were blocked with goat serum and primary antibodies were applied for 2 hrs. Sections were thoroughly rinsed and processed with DakoCytomation EnVision System Kit (HRP/Rabbit) (Dako Corp., USA). A diaminobenzidine tetrahydrochloride (DAB) solution was then used. All slices were then counterstained with hematoxylin. Sections treated with phosphate buffer solution (PBS) instead of primary antibodies were chosen for negative control. Evaluation of TFPI-2 expression level was performed semi-quantitatively. All slides were captured using camera (MV-CP410, Panasonic, Japan) coupled to a microscope (BH2, Olympus, Japan). All images were analyzed digitally using the IMS cell image processing system (Shenteng Tech., Shanghai, China). When backgrounds were filtered, the positive regions were observed using the optical density (OD). The positive area and OD of TFPI-2 positive cells were determined by measuring three randomly selected microscopic fields (×400 magnification) for each slide. The IHC index was defined as the average integral optical density (AIOD) (AIOD = positive area OD/total area). Staining and evaluation for TP53 and Ki67 were reported in our previous studies [13,16,17].

In situ apoptosis detection

The *in situ* apoptosis detection kit (Roche, Switzerland) was used to evaluate the apoptotic bladder cancer cells within paraffinized samples. Preparation of the slides was similar with IHC procedures and 0.3% hydrogen peroxide processed samples were further treated with DNA protease K and subsequently with chilled PBS. Samples were rinsed 3 times and were then treated with buffering solution. After centrifuge, TDT and Biotin-11-dUTP were mixed gently and diluted with buffering solution, which was then added to the samples. All sections were then dipped in diluted SSC solution for 15 min and were thoroughly rinsed. After blockade, samples were processed with working solution consisting of 1:50 Avidin-HRP for 60 min at room temperature. Slides were finalized with DAB development after rinses. Granular DAB within cells were hallmarks for positivity and were quantified using the apoptotic index, which was calculated by the mean of 3 percentages, each being the percentage of positive cells with a certain vision field under 400× magnification.

Quantitative RT-PCR and western blotting

The standard qRT-PCR and western blotting procedures followed, as previously reported [18]. After concentration was determined, RNAs were converted to cDNAs, which were further subjected to the ABI 7500 reactor (ThermoFisher Scientific, USA) in 20μl system. Forward and reverse primers of TFPI-2 and internal control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were TFPI-2 F 5'-CTTGCGACGATGCTTGCTG-3' and R 5'- ACACCCACCGGAAAAGAATTT-3'; GAPDH F 5'- ATGGGGAAGGTGAAGGTCG-3' and R 5'-GGGGTCATTGATGGCAACAATA-3'. For each sample, the average value of threshold cycle was normalized to GAPDH level with the formula 2^{-Ct}. For western blotting, endogenous GAPDH was used as an internal reference. Cell lysates were separated with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, which were subsequently blocked for 4 hrs. The membranes were then incubated with TFPI-2 polyclonal antibody (ImmunoWay,USA) overnight at 4°C. Samples were finalized by enhanced chemiluminescence (EnVision, Dako, Denmark) to exhibit bands.

Statistics

The SPSS version 16 software was used for statistical analyses. All data were presented as mean ± standard deviation (SD). For comparisons between 2 groups, the Mann-Whitney U test was used. For comparisons among 3 or more groupings, the Kruskal-Wallis test was applied. Correlations were studied using the Pearson test. P <0.05 was accepted as statistically significant.

Results

Clinicopathologic correlation and TFPI-2

The clinicopathological parameters are summarized in Table 1. There were 24 patients with bladder cancer included in the current study. The mean age of the patients was 64.28±11.2 years. The IHC index of TFPI-2 was significantly decreased in tumors with higher grade and stage. TFPI-2 expression was not altered in tumors with different sizes (Table 1). TFPI-2 expression was not associated with single or multiple occurrences (Table 1). Western blotting of TFPI-2 protein detected in the corresponding samples subjected to IHC showed a consistent protein level as demonstrated in the IHC index (Figure 1). The staining of TFPI-2 was cytosolic (Figure 1). Also, the quantitative PCR showed high consistency between mRNA expression and protein level of TFPI-2 (data not shown).

Correlation between TFPI-2, apoptosis, Ki-67, TP-53

The apoptosis was profiled using the apoptosis index based on the granular staining (Figure 2). The correlation analysis revealed that TFPI-2 expression was positively correlated with apoptosis (r=0.652, p<0.001). Also, the expression of TFPI-2 was not correlated with that of p53 (r=-0.274, p=0.195). TFPI-2 expression was however negatively correlated with that of Ki67 (r = -0.588, p=0.003, Figure 3).

Discussion

TFPI-2, a member of the Kunitz-type serine proteinase inhibitor family, is inversely related to an increasing grade of malignancy. Our previous study [18] showed that the expression of TFPI-2 and invasiveness of renal cell carcinoma had a negative correlation and overexpression of TFPI-2 could induce tumor cell apoptosis. Lower expression of TFPI-2 in renal cell carcinoma was partly due to hypermethylation of the gene promoter. Hypermethylation of TFPI-2 promoter CpG

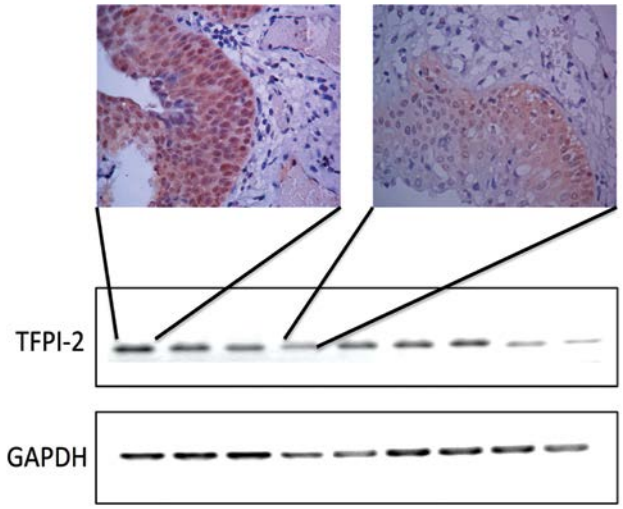

Figure 1. Representative immunohistochemical staining of TFPI-2 in bladder cancer. Staining intensity was consistent with the corresponding lane in Western blotting (original magnification×400).

Table 1. Clinicopathological parameters and TFPI-2 level in urinary bladder cancer.

Parameters		*N*	*%*	*TFPI-2*		*p value*
				Mean	*SD*	
Grade	PUNLMP	7	29.2	265.8	167.7	< 0.001
	Low	11	45.8	90.7	38.2	
	High	6	25	16.3	17.2	
Stage	Ta	12	50	198.9	140.0	< 0.001
	T1	7	29.2	58	16.4	
	T2	5	20.8	11.6	14.2	
Size (cm)	≥3	6	25	109	46.4	0.536
	<3	18	75	122.1	146.9	
Multifocality	No	18	75	96.8	77.9	0.378
	Yes	6	25	184.8	219.3	
Gender	Male	16	66.7	98.2	80.4	0.275
	Female	8	33.3	160	193.1	

PUNLMP = papillary urothelial neoplasm of low malignant potential

islands in human cancers and cancer cell lines was demonstrated to be responsible for downregulation of mRNA encoding TFPI-2 and decreased or abolished synthesis of TFPI-2 protein during cancer progression [19-21]. The finding was associated with increased cancer growth and invasion. TFPI-2 methylation was detected in 29% of metastatic melanoma specimens [22,23], while it was not detected in any of the primary melanoma specimens [22]. Immunochemically, all melanoma specimens with TFPI-2 promoter methylation lacked immunoreactivity for TFPI-2. In non-small cell lung cancer (NSCLC), decreased TFPI-2 expression and hypermethylation were more frequently associated with stage III and IV of disease [24]. Approximately one half of NSCLC cases with silenced TFPI-2 gene were lymph node-positive [25]. TFPI-2 methylation was observed in all cases of oesophageal carcinoma cell lines [26]. Aberrant methylation of TFPI-2 was detected in as many as 73% of pancreatic cancer xenografts and primary pancreatic adenocarcinomas, and was more likely in old patients with pancreatic cancer. Hypermethylation of TFPI-2 was significantly associated with progression of primary pancreatic ductal neoplasms [27]. The aberrant methylation rate of TFPI-2 in pure pancreatic juice aspirated endoscopically from patients was as high as 100% ; promoter methylation of TFPI-2 in the pure pancreatic juice may be an effective marker in diagnosis and progression of pancreatic cancer [28]. Since TFPI-2 promoter methylation was detected in 80% of hepatocellular carcinoma cell lines and 47% of human hepatocellular carcinoma, leading to overexpression of TFPI-2 which significantly suppressed the proliferation and invasiveness of hepatocellular carcinoma cells, TFPI-2 proved to be a candidate tumor suppressor gene in human hepatocellular carcinoma [29].

The current study has shown that TFPI-2 expression was decreased in bladder cancer with higher grade and stage but not with tumor size or multifocality. These results indicated that decreased TFPI-2 could be associated with the inherent invasiveness of the tumor. Also we have shown that expression of TFPI-2 was positively correlated with apoptosis, implying the pro-apoptotic role of TFPI-2 in bladder cancer. This was similar to our observation in renal cell carcinoma (RCC) that TFPI-2 was also associated with apoptosis in RCC [18]. Our group has now been pursuing this lead to further elaborate the detailed mechanistic mediation of TFPI-2 in apoptosis. We therefore speculate that hypermethylation could be a mechanism by which TFPI-2 is downregulated in bladder cancer. Also, we have shown that TFPI-2 expression was negatively correlated with Ki67 index. The proliferative activity of tumors determined by Ki67 labelling index was found to correlate with aggressive behavior of many tumor types including bladder cancer [16,17]. Although tumor grade and stage are considered signs of aggressive behavior for bladder cancer, several reports in the literature describe a correlation between Ki67 labelling index with well-known prognostic factors, such as grade and stage [16,17].

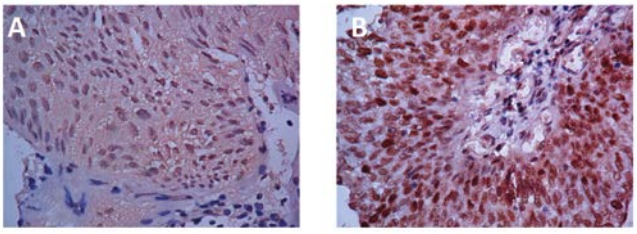

Figure 2. Representative immunohistochemical staining of Biotin-11-dUTP, indicative of apoptotic bladder cancer cells, showing **A**: bladder cancer sample with relatively lighter granular staining within cells that are hallmarks for apoptotic activity in contrast with **B**: much stronger staining indicative for apoptosis (original magnification ×400).

Our study has limitations. First, we did not include normal bladder urothelium as a control for comparison. This was due to insufficient sample quality for IHC evaluation. Second, the sample size of the current study is still small. Validation with larger sample size is warranted. Third, tissue sample in the current study should be evaluated for methylation status of TFPI-2, and relevant studies are now ongoing by our group.

In conclusion, this study has shown that TFPI-2 decrease could be an indicator for disease progression of bladder cancer and modalities that could upregulate TFPI-2 could be a novel therapy for bladder cancer.

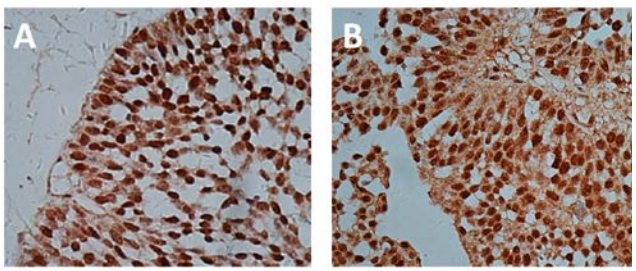

Figure 3. A: Representative immunohistochemical staining in bladder cancer sample showing strong positivity of TFPI-2, in contrast with **B**: immunohistochemical staining showing strong TP53 expression with similar intensity in the same sample.

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Conflict of interests

The authors declare no conflict of interests.

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