RESEARCH ARTICLE

Prognostic Value of T Cell Immunoglobulin Mucin-3 in Prostate Cancer

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Abstract

Background: Optimal treatment for prostate cancer remains a challenge worldwide. Recently, T cell immunoglobulin mucin-3 (TIM-3) has been implicated in tumor biology but its contribution prostate cancer remains unclear. The aim of this study was to investigate the role of TIM-3 as a prognostic marker in patients with prostate cancer. Methods: TIM-3 protein expression was determined by immunohistochemistry and Western blotting in 137 prostate cancer tumor samples and paired adjacent benign tissue. We also performed cell proliferation assays using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl- 2H tetrazolium bromide (MTT) and cell invasion assays. The effects of small interfering RNA (siRNA)-mediated knockdown of TIM-3 (TIM-3 siRNA) in two human prostate cancer cell lines were also evaluated. Results: TIM-3 expression was higher in prostate cancer tissue than in the adjacent benign tissue ($P<0.001$). High TIM-3 expression was an independent predictor of both recurrence-free survival and progression-free survival. TIM-3 protein was expressed in both prostate cancer cell lines and knockdown suppressed their proliferation and invasion capacity. Conclusions: TIM-3 expression is associated with a poor prognosis in prostate cancer. Taken together, our resutlts indicate that TIM-3 is a potential prognostic marker in prostate cancer.

Keywords: TIM-3 - prostate cancer - prognosis - biomarker

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Introduction

Prostate cancer is one of the most prevalent malignancies in men and the second most frequent cause of male cancer-related death (Jemal et al., 2010). It is a clinically heterogeneous-multifocal disease and the incidence is continuously rising (Sugiyama et al., 2013). Carcinogenesis and the mechanisms influencing the progression and prognosis of prostate cancer involve a multistep process (Xu et al., 2012). Improved diagnostic and prognostic biomarkers for prostate cancer are needed to prevent overtreatment of indolent tumors, and to ensure early detection of aggressive Prostate cancer that requires treatment (Facompre et al., 2009).

T cell immunoglobulin mucin-3 (TIM-3), a membrane protein, plays a pivotal role in immune regulation (Zhuang et al., 2012). Current studies have demonstrated a strong correlation between TIM-3 expression and tumor-associated immune suppression. TIM-3 can be upregulated by transforming growth factor β (TGF-β) treatment and contributes to the survival of melanoma (Wiener et al., 2007). Targeting TIM-3 pathways may reverse T cell exhaustion and restore anti-tumor immunity (Sakuishi et al., 2010). In melanoma patients, upregulation of TIM-3 is associated with tumor antigen-specific CD8+ T cell dysfunction (Fourcade et al., 2010). In addition, TIM-3 suppresses activation of CD4+ T lymphocytes through the activation of the interleukin-6-STAT3 pathway and also facilitates the establishment of lymphoma immune tolerance (Huang et al., 2010). However, the role of TIM-3 in prostate cancer remains largely unknown.

The goal of this study was to examine the significance of TIM-3 in prostate cancer. We detected the expression of TIM-3 protein in primary prostate cancer tumors from a large population of patients. In vitro studies were also carried out to investigate the role of TIM-3 expression in prostate cancer cell lines.

Materials and Methods

Patients and tissue specimens

Specimens of prostate cancer and paired benign tissues (n=137 per group) were collected from patients in the Urology Department of our hospital between January 2006 and December 2011. All patients were newly diagnosed as prostate cancer by histopathology, who did not receive any treatment previously. Benign tissues were surgically excised from the same patients and confirmed as normal by histology. All of the tissues were obtained immediately during the operation of transurethral resection prostate and suprapubic prostatectomy. None of the patients recruited in this study had chemotherapy or radiotherapy before

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the surgery. The pathological diagnosis was performed preoperatively and confirmed postoperatively. Informed consent was obtained from all patients. Recurrence or metastasis of prostate cancer and disease-related death were obtained from the review of medical records. Tumor stage and nuclear grading were classified according to the 2010 tumor, node and metastasis (TNM) stage system. This study was approved by the Institutional Review Board of Medical School of our hospital.

Immunohistochemical and Western blot Detection of TIM-3

Paraffin-embedded tissue sections were subjected to immunohistochemical analysis as described previously (Geng et al., 2006). Anti-TIM-3 antibody (Abcam) was used in the present study. For semiquantitative evaluation, intensity of staining was designated as 0, 1+, 2+ and 3+. These data were compared between various groups. In addition, Western blot was used to prove the specificity of anti-Tim-3 antibody and confirm the expression of Tim-3.

Cell lines

Two human prostate cancer cell lines were obtained directly from the NCI (DU145) or ATCC (LNCaP) were purchased from the American Type Culture Collection. Human benign prostate epithelial cell line BPH-1 was used as normal control. Cells were cultured as previously described (Abildgaard et al, 2012).

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using Trizol and reverse transcribed using SYBR® Quantitative PCR Kit (Invitrogen). Primers for TIM-3 (5'-TCCAAGGATGCTTA CCACCAG-3', 5'-GCCAATGTGGATATTTGTGTTAGA TT-3') were used and the transcript was normalized to GAPDH (forward, 5'-GACCCTTCATTGACCTC-3', reverse, GCAATG CCAGCCCCAG) transcript levels.

Western blot

Cell lysates were resolved using a 10% polyacrylamide gel in a sodium dodecyl sulfate buffer by electrophoresis. After being transferred onto nitrocellulose membrane, the blots were incubated with anti-TIM-3 antibody (Abcam). Binding of TIM-3 antibody was revealed by chemiluminescence after incubation with horseradish peroxidise-conjugated goat anti-mouse antibody (Bio-Rad Laboratories). β-Tubulin (Abcam) was used as the internal control.

Cell proliferation assay

To evaluate the effects of TIM-3 inhibition on the proliferation of prostate cancer cell lines, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay was carried out. TIM-3 siRNA or control siRNA (Santa Cruz) was chosen to selectively inhibit TIM-3 activity. Briefly, 1×10^5 cells of each group were plated per well in 24-well plates. Both RCC cell lines were incubated with various concentrations of TIM-3 siRNA (0, 0.4, 0.8, 1, 1.2, and 1.5 μM). After 48h incubation, the MTT substrate (Sigma, 5 mg/mL in phosphate buffered saline) was added to each well, and the cells were incubated at 37°C for four hours. Following the elimination of the culture medium, the cells were dissolved in 1mL of dimethyl sulfoxide. The optical density was measured using a microplate reader at 490 nm wavelength. Each experiment was repeated three times.

Cell invasion assay

To evaluate the influence of TIM-3 inhibition on invasive capacity of prostate cancer cell lines, we used a CHEMICON cell invasion assay kit (Millipore) according to the manufacturer's instructions. DU145 and LNCaP cells (1×10^5) were seeded in the upper chamber in a 300 μL of serum free media incubated with treatment of a 1.0 μM of TIM-3 siRNA, and a 500 μL of 10% FBS medium was placed in the lower chamber as a chemo-attractant. The cells were incubated for 48 hours at 37°C in a 5% CO_2 chamber. This was followed by the removal of the cells on the upper surface of the membrane using a cotton swab. The cells, infiltrating to the lower surface of the membrane, were fixed with methanol and stained for 20 minutes, and then the number was counted from 10 random microscopic fields at a ×100 magnification.

Statistical analysis

SPSS 17.0 and GraphPad Prism 5.0 were used for data analyses. Prognosis was assessed using Kaplan-Meier survival curves and Cox univariate and multivariate analysis of according to TIM-3 expression. The Spearman correlation was calculated between the expression levels of TIM-3 in prostate cancer tissues. Differences were considered statistically significant when $p<0.05$.

Results

Clinicopathological features

The clinicopathological characteristics patients with prostate cancer are summarized in Table 1. The mean age of 137 patients were 57 years, and ranged from 29 to 81 years. During the follow-up period, there were 24 cases (17.5%) of pulmonary metastasis and eight cases (5.8%) of bone metastasis. The median follow-up period was 68 months (range 3 to 115 months).

Correlations between TIM-3 and clinicopathological features

An analysis of TIM-3 expression was performed for 137 prostate cancer tumor samples. The staining intensity was scored from 0 to 3+ (Figure 1). While absent or weakly expressed in benign prostatic glands (Figure 1A), TIM-3 was clearly up-regulated in prostatic intraepithelial neoplasia (PIN) (Figure 1B) and in invasive carcinoma TIM-3 was expressed in various fractions and intensities (Figure 1C-E). To evaluate the statistical significance, the cases were redistributed into two groups according to the degree of their expression: the TIM-3-low group (0, 1+ and 2+, n=109, 79.6%) and the TIM-3-high group (3+; n=28, 20.4%). The correlations between TIM-3 immunoreactivity and clinicopathological variables are summarized in Table 1. The TIM-3 expression was statistically higher in prostate cancer and also correlated with the pulmonary metastasis based on follow-up

Table 1. Correlations of Clinicopathologic Features with Tim-3 Expression

	Cases (n)	TIM-3 expression (n=137)		*p*
		Low (n=109)	High (n=28)	
T				
1	87	70	17	0.427
2	23	20	3	
3	26	18	8	
4	1	1	0	
N				
N0	134	107	27	0.499
N1	3	2	1	
M				
M0	124	100	24	0.303
M1	13	9	4	
TNM stage				
I	86	69	17	0.581
II	18	16	2	
III	19	14	5	
IV	14	10	4	
Nuclear grade				
1	9	8	1	0.744
2	61	48	13	
3	49	40	9	
4	18	13	5	
Lung metastasis during follow-up				
Absent	113	94	19	0.022
Present	24	15	9	
Bone metastasis during follow-up				
Absent	129	104	25	0.36
Present	8	5	3	

T, size or direct extent of the primary tumor; T1, T2, T3, T4, size and/or extension of the primary tumor; N, degree of spread to regional lymph nodes; N0, tumor cells absent from regional lymph nodes; N1, regional lymph node metastasis present; M, presence of distant metastasis; M0, no distant metastasis; M1, metastasis to distant organs

(p=0.022). In addition, Western blot proved the specificity of Tim-3 antibody and confirmed the protein expression of Tim-3 (Figure 2).

TIM-3 expression and prognosis

The recurrence-free and progression-free survival were significantly shorter in the TIM-3-high group as compared with the TIM-3-low group (p=0.038 and p=0.031, respectively) (Figure 3 A, B). On univariate analysis, the TIM-3 expression had a significant association with recurrence-free or progression-free survival, TNM stage, and nuclear grade (all p<0.01). On multivariate analysis, TIM-3 expression was an independent predictor of both recurrence-free [risk ratio = 2.08; 95% confidence interval (CI) 1.07-4.16; P=0.007] and progression-free survival [risk ratio = 2.23; 95% confidence interval (CI) 1.42-4.57; P<0.001].

Effect of TIM-3 siRNA on tumor cell proliferation and invasion

Western blot analysis showed positive TIM-3 expression on both DU145 and LNCaP cell lines (Figure 4A). MTT assay was performed to assess the effects of TIM-3 siRNA on the viability and proliferation of the cell lines. The effects of TIM-3 siRNA were confirmed by qRT-PCR and Western blot (Figure 4 B, C).

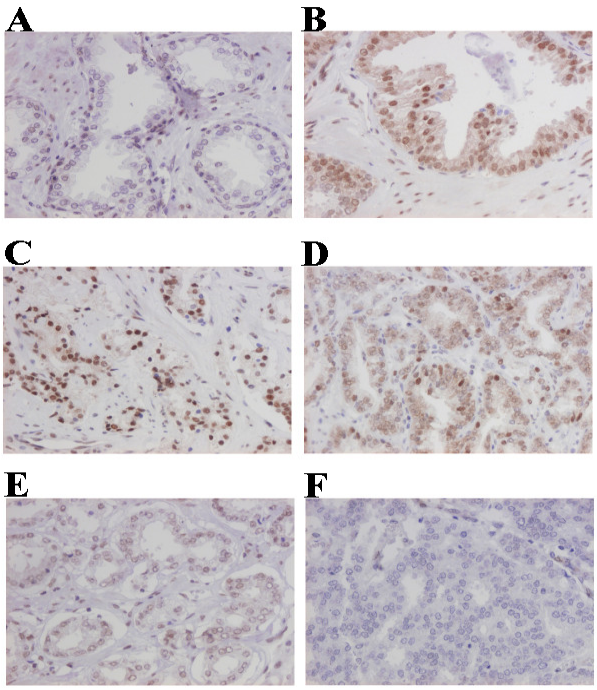

Figure 1. Immunohistochemical Findings of TIM-3 in Normal Prostatic Epithelium, Prostatic Intraepithelial Neoplasia and in Prostate Cancer. Immunohistochemical images of TIM-3 expression in (A) normal prostatic epithelium, (B) prostatic intraepithelial neoplasia and invasive prostate cancer, ranging from (C) +3, (D) +2, (E) +1 to (F) 0 (negative expression)

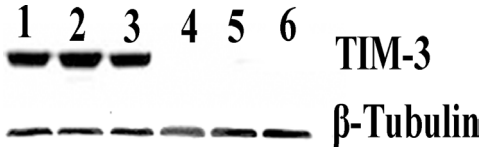

Figure 2. Survival Analysis in Prostate Cancer. Kaplan-Meier curves of recurrence-free (A) and progression-free (B) survival in 137 patients with prostate cancer depending on the T cell TIM-3 expression

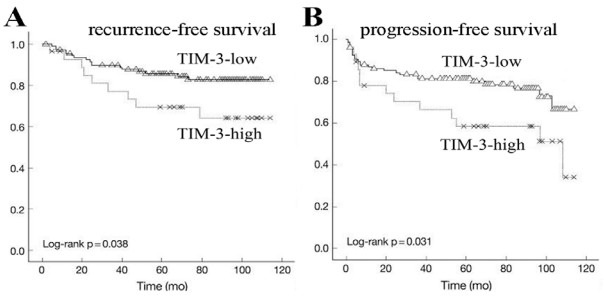

Figure 3. Western Blot Analysis of T Cell Immunoglobulin Mucin-3 (TIM-3) in Prostate Cancer (Lane 1-3) Compared with Adjacent Benign Tissue (Lane 4-6). The internal control (β-Tubulin) were used

The TIM-3 siRNA-treated group revealed a dose-dependent suppression of cell proliferation (Figure 4 D, E). And results showed that TIM-3 siRNA inhibited the invasion capacity of both DU145 and LNCaP prostate cancer cell lines (Figure 4 F, G). These results demonstrated that TIM-3 siRNA down-regulated both the proliferation and invasion capacity of prostate cancer cell lines.

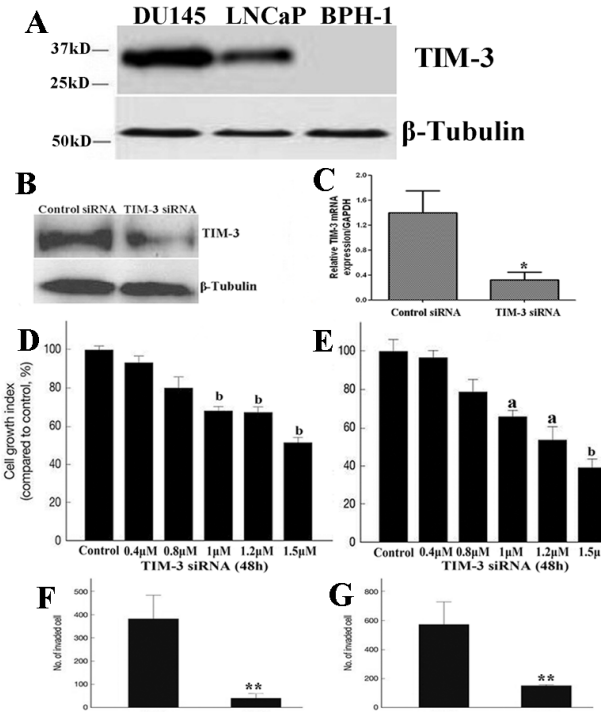

Figure 4. Western Blot Analysis of T Cell Immunoglobulin Mucin-3 (TIM-3) and Cell Proliferation and Invasion by TIM-3siRNA. A. Both DU145 and LNCaP cell lines expressed TIM-3 protein. The human prostate epithelial cell line BPH-1 and an internal control (β-Tubulin) were used. Western blot (B) and real time PCR (C) proved the downregulation of Tim-3 expression at mRNA and protein levels by 1.0 μM siRNA for 48 h (*p<0.01). MTT assay shows growth inhibition of DU145 (D) and LNCaP (E) cell lines after treatment with various concentrations of TIM-3 siRNA for 48 h. Data presents as the percentage of control and shows mean ± standard deviation from three independent experiments(ap<0.05, bp<0.01). Histograms show the reduced invasive capacity of DU145 (F) and LNCaP (G) cell lines after treatment with a 1.0 μM TIM-3 siRNA for 48 h. Data are presented as the mean±standard deviation from three independent experiments (**p<0.01)

Discussion

To date, there have been limited reports on the importance of the TIM-3 in prostate cancer. A recent study suggests that TIM-3 polymorphisms are associated with renal cell carcinoma (Cai et al., 2012). Here, we demonstrated positive TIM-3 expression in tumor tissues from patients with prostate cancer, whereas paired normal tissues showed negative or weak expression of TIM-3. In addition, we found a statistically significant correlation between TIM-3 protein expression in tumor tissues and clinicopathologic parameters. More importantly, we found that the survival of patients with high TIM-3 expression was significantly lower than that of those patients with low TIM-3 expression. The univariate and multivariate analyses revealed that TIM-3 status in tumor tissues was an independent prognostic factor of prostate cancer.

Wiener et al. (2007). found that Tim-3 was expressed not only in mast cells around melanomas, but also in tumor cells in tissue sections and human melanoma cell lines, in line with our study. Studies have also identified Tim-3 expression on leukemia stem cells in patients with acute myeloid leukemia (Kikushige et al., 2010; Jan et al., 2011). The link between Tim-3 expression and tumor cell itself has not yet been well defined. To further investigate the role of the TIM-3 pathway in prostate cancer, in vitro studies were carried out in our study. Positive TIM-3 expression was identified in both prostate cancer cell lines. One striking finding was that exposure to TIM-3 siRNA resulted in significant inhibition of cell proliferation and invasion. How is Tim-3 involved in tumor biology? In vitro, Tim-3 can activate the interleukin-6 (IL-6)-STAT3 pathway. According to published data (Yun et al., 2012; Yang et al., 2013). IL-6-STAT3 pathway plays an important role in tumor growth. IL-6-STAT3 signaling is involved in tumor growth and metastasis of human hepatocellular carcinoma (Yang et al., 2013). In addition, DNA damage induces the IL-6-STAT3 signaling pathway, which has growth-promoting functions in human tumors (Yun et al., 2012). Based on these previous findings and our results, we hypothesize that Tim-3 might facilitate tumor metastasis through the IL-6-STAT3 pathway.

Taken together, our work confirms that TIM-3 protein expression is associated with poor survival in prostate cancer. Based on in vitro activity of TIM-3 siRNA in prostate cancer cell lines, TIM-3 might be a good target for this disease. Thus, further evaluation of TIM-3 in a larger scale of prostate cancer samples is warranted.

Acknowledgements

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