Significance of the detection of TIM-3 and FOXJ1 in prostate cancer
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Summary

**Purpose:** This study sought to identify and evaluate the diagnostic value of T-cell immunoglobulin domain and mucin-3 (TIM-3) and forkhead box protein J1 (FOXJ1) expression in prostate cancer.

**Methods:** Thirty prostate cancer patients and 30 individuals with benign prostatic hyperplasia diagnosed and treated at the Central Hospital of Enshi Autonomous Prefecture between March 2016 and October 2016 were selected for this study. The expression of TIM-3 and FOXJ1 in patient prostate tissue was detected by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). TIM-3 and FOXJ1 expression diagnostic value for prostate cancer was analyzed by using the receiver operating curve (ROC).

**Results:** Expression of TIM-3 and FOXJ1 in prostate cancer tissues was significantly higher than those in normal prostate tissues (p<0.05), and expression of TIM-3 and FOXJ1 in prostate cancer tissues were positively correlated with Gleason score and clinical stage (p<0.05). However, the expression of the two proteins were not correlated with age, PSA level, pathological type, or the maximum tumor diameter (p>0.05). ROC analysis indicated that TIM-3 mRNA could be used to diagnose prostate cancer with an accuracy of 0.824, a sensitivity of 85.9% and a specificity of 91.2%, while the diagnostic accuracy, sensitivity, and specificity of FOXJ1 were 0.843, 86.3%, and 82.7%, respectively.

**Conclusion:** TIM-3 and FOXJ1 exhibited abnormally high expression levels in prostate cancer, and can therefore be important indicators for the diagnosis of this disease.

**Key words:** Forkhead box protein J1 (FOXJ1), prostate cancer, T cell immunoglobulin domain and mucin-3 (TIM-3)

Introduction

The incidence and mortality of prostate cancer ranks second among males with cancer in China. Local radical resection during the early stage is an important method for improving prognosis. Metastases of prostate cancer are mainly in the liver and bone [1]. Prostate specific antigen (PSA) levels provide great assistance in early screening, diagnosis, and follow-up, but 10-30% of prostate cancer patients have negative PSA results [2]. Recent studies have shown that the T-cell immunoglobulin domain and mucin-3 (TIM-3) played an immune-escape role in the pathogenesis of many tumors [3]. TIM-3 is an immunoglobulin expressed primarily on the surface of mature differentiated Th1 cells, and is less expressed in Th2 cells [4]. TIM-3 was also involved in the phagocytic apoptotic cell process by acting as a phosphatidylserine receptor [5]. The forkhead box protein (FOX) transcription factor family has been shown to play a key role in the development and progression of tumors in the stomach and liver [6,7]. FOXJ1 is involved in embryonic development and differentiation, cilia formation, and autoimmune response processes [8]. FOXJ1 could suppress tumor development in meningioma and ovarian cancers [9], while it seemed to promoter liver cancer development [10]. The heterogeneity of FOXJ1 expression in different tumors may be related to tumor...
microenvironments [11]. This study was designed to analyze the expression and diagnostic value of TIM-3 and FOXJ1 in prostate cancer.

Methods

Subject Information

Thirty prostate cancer patients who were diagnosed for the first time and treated at the Central Hospital of Enshi Autonomous Prefecture were continuously enrolled from March 2016 to October 2016. The patient age ranged from 48-72 years (mean 55.6 ± 12.3). Serum PSA levels ranged between 10-40 ng/ml (mean 25.8 ± 11.2). There were 23 cases of adenocarcinoma, 5 cases of adenosquamous carcinoma, and 2 cases of urothelial carcinoma. Five patients had a Gleason score between 2 to 4 (well differentiated), 19 had a Gleason score of 5 to 7 (moderately differentiated), and 6 had a Gleason score of 8 to 10 (poorly differentiated). In terms of clinical stage, the number of patients in stages I, II, III, and IV were 4, 16, 8, and 2, respectively. Thirty patients with benign prostatic hyperplasia aged 45-70 years (mean 54.8 ± 13.2) were also selected. All patients provided informed consent. Patients with previous prostate surgery, radiotherapy/chemotherapy history, or autoimmune diseases were excluded.

Research Methods

The expression of TIM-3 and FOXJ1 was detected by immunohistochemistry and RT-PCR. The relationships between TIM-3/FOXJ1 expressions and clinical characteristics were analyzed. The diagnostic value of TIM-3 and FOXJ1 for prostate cancer was assessed by ROC analysis.

Immunohistochemistry

Slides with 5 μm-thick tissue sections were dewaxed, hydrated, and underwent antigen retrieval. They were then incubated with mouse anti-human TIM-3 and FOXJ1 monoclonal antibodies (Beyotime Biotechnology, Nantong, Jiangsu, China; working concentration 1: 3000) in a wet box at 4°C overnight. After washing the slides with phosphate buffered saline (PBS), rabbit anti-mouse polyclonal secondary antibody was added (Beyotime Biotechnology, Nantong, Jiangsu, China; working concentration 1:1000) and the incubation was performed in a wet box at 27°C for 20 min. After PBS washing and diaminobenzidine (DAB) staining, the slides were cover-slipped, sealed, and observed under optical microscope (Olympus, Tokyo, Japan). The results were evaluated by a semi-quantitative method based on staining intensity and the proportion of stained cells. Yellow to dark brown staining in the cytoplasm or nucleus was considered positive staining. The grading based on the staining intensity was: 0 points for no positive staining, 1 point for weak staining, 2 points for medium staining, and 3 points for strong staining. The grading based on positive cell ratio was: 0 points for ≤ 5%, 1 point for 6-25%, 2 points for 26-0%, 3 points for 51-75%, and 4 points for > 75%. The final results were based on the products of the above two gradings: 0 to 5 was considered negative while 4 to 12 was considered positive.

RT-PCR

Total RNA was extracted from cells using Trizol reagent (Sigma, St. Louis, MO, USA), and concentration and purity was determined by ultraviolet spectrophotometer (Santa Cruz, CA, USA). cDNA was synthesized using a reverse transcription kit (TaKaRa, Kusatsu, Shiga, Japan). The primers were designed based on the sequences of TIM-3 and FOXJ1 obtained from Gene Bank and were synthesized by Sango Biotech (Shanghai, China). Primer sequences and amplicon sizes were as follows: TIM-3: (F) 5’-GATAACGCTCAATCCGGTC-3’, (R) 5’-GCATGTAGTCAAGAATTG-3’, 352 bp; FOXJ1 (F): 5’-GATAACGCTCAATCCGGTC-3’, (R) 5’-GAACTCATCAGAGGGTTCAAT-3’, 315 bp; GAPDH (F): 5’-CCGGAAGAGATGACCCAGAT-3’, (R) 5’-GACTGTGTTGGCGTGATAC-3’, 225 bp. Each reaction contained 2 μl cDNA, 3 μl each forward and reverse primers, 0.5 μl Taq polymerase, 1 μl dNTPs, 5 μl MgCl2, 5 μl 10 × Buffer, and water was added for a final volume of 50μl (all PCR reagents were purchased from GE Healthcare, Chicago, IL, USA). The reaction conditions were: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 60 s, then 72°C for another 10 min. PCR products were separated by 2% agarose gels and gel images were captured by a gel imaging analysis system (Media Cybernetics, Rockville, MD, USA). The results were analyzed by comparing the gray values of the bands.

Statistics

Statistical analyses were performed using SPSS20.0 software (SPSS, Chicago, IL, USA). Quantitative data were expressed as mean ± standard deviation. Independent sample t-test was used for between-group comparisons. Count data were expressed as number of cases or %, and comparisons between groups were examined by χ2 test or Fisher exact probability test. The diagnostic values of TIM-3 and FOXJ1 for prostate cancer were analyzed using ROC curves. p<0.05 was considered statistically significant.

Results

Immunohistochemical detection of CD40 in patient tissue samples

The positive expression rates of TIM-3 and FOXJ1 in prostate cancer tissues were significantly higher than in hyperplastic prostate tissues (p<0.05, Figure 1, Table 1).

RT-PCR results

The expression of TIM-3 and FOXJ1 mRNA in prostate cancer tissues was significantly higher than in hyperplastic prostate tissues (p<0.05, Figure 2).
Correlation of TIM-3 and FOXJ1 expression and clinical characteristics of prostate cancer

TIM-3 and FOXJ1 expression in prostate cancer tissues were correlated with Gleason score and clinical stage, and a positive correlation was noted for both (p<0.05). No correlation of TIM-3 and FOXJ1 expression was noted with age, PSA level, pathological type, or the maximum tumor diameter (p>0.05, Table 2).

ROC analysis

ROC analysis showed that TIM-3 could be used to diagnose prostate cancer with an accuracy of 0.824 (indicated by the area under the curve, 95% CI=0.786-0.932, p=0.025), a sensitivity of 85.9%, and a specificity of 91.2%. The threshold value of TIM-3 mRNA for prostate cancer was 0.3425. The diagnostic accuracy of FOXJ1 mRNA was 0.843 with 95% CI=0.792-0.945 and p=0.023. The diagnostic sensitivity and specificity of FOXJ1 were 86.3% and 82.7%, respectively and the threshold value was 0.2352 (Figure 3).

Discussion

A previous study [12] has shown that TIM-3 expression in peripheral CD4+ and CD8+ T cells of prostate cancer patients was significantly increased, affecting Th1 cell transport. The binding of its ligand galectin-9 could induce peripheral immune tolerance. TIM-3 was highly expressed in a variety of malignant tumors of epithelial origin, such as urothelial bladder carcinoma and gastric cancer, and it was closely related to progression and prognosis of the tumors [13]. The co-expression of TIM-3 and the programmed death receptor 1 (PD-1) in the tumor microenvironment was considered a hallmark of T-cell failure [14]. Blocking the PD-1/B7-H1 pathway could significantly reverse T-cell failure and enhance the anti-tumor immune activity of T cells, and was expected to become a tumor-immune intervention target [15]. TIM-3 and cytotoxic T lymphocyte-associated antigen 4 had negative immune-checkpoint regulating functions, which protected the host from autoimmune responses.
and limited the anti-tumor activity of T cells. TIM-3 is a type I transmembrane glycoprotein, and the variable immunoglobulin domain at the membrane side was the key structure of its inhibitory functions [16].

FOX proteins could activate the transcription of their target genes through recruitment of coactivators. For example, FOXM1b could recruit cyclin-dependent kinase complexes via the LXL motif in the transcriptional activation domain, thereby promoting the binding of the coactivator p300/CPB to promoters to initiate transcription [17]. The transcriptional activity of FOX was regulated at multiple levels by gene transcription, mRNA stability, protein stability, and protein-protein interactions. The FOX family played an end-effector role in a variety of signal transduction pathways such as the Sonic-Hedgehog pathway, MAPK pathway, Wnt/β-catenin pathway, and TGF-β pathway [18]. These are involved in embryonic development, cell proliferation, differentiation, migration, invasion, apoptosis, and autophagy [19]. FOXJ1 was found to be an important immunoregulatory transcription factor involved in the immune tolerance of CD4+ T cells [20]. In vitro, FOXJ1 could inhibit the transcription of NF-kB, promote the expression of anti-apoptotic proteins, cytokines, chemokines, and cell adhesion molecules, and induce malignant cell proliferation, immune escape, invasion, and metastasis [21]. FOXJ1 was also a key downstream regulator in the regulation of ovarian cancer invasion and metastasis by the stem cell transcription factor NANOG [22].

This study indicated that the protein and mRNA expression levels of TIM-3 and FOXJ1 in prostate cancer tissues were significantly higher compared to the hyperplastic prostate tissues. The positive expression of TIM-3 and FOXJ1 were positively correlated with Gleason score and clinical stage, but not with age, PSA level, pathological type, or maximum tumor diameter. ROC analysis showed that TIM-3 and FOXJ1 mRNA could diagnose prostate cancer with high sensitivity, specificity, and accuracy. These results suggested that abnormally high TIM-3 and FOXJ1 expression could be an important indicator in the diagnosis of prostate cancer.

This study provided two potentially important indicators for the early diagnosis and prognosis evaluation of prostate cancer, as well as important targets for the immunotherapy of prostate cancer. The shortcoming of this research was the small sample size. In addition, the specific mechanisms of TIM-3 and FOXJ1 in the regulation of prostate cancer development and progression were not well elucidated.

**Conflict of interests**

The authors declare no conflict of interests.
References