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Original Article

Expression of p-FOXO3/FOXO3 in bladder cancer and its correlation with clinicopathology and tumor recurrence

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Abstract: Background: Survey for more accurate biomarkers for predicting and preventing the future recurrence in high risk patients is urgently needed. The transcription factor forkhead box-O3 (FOXO3) is a well-established tumor suppressor. Its phosphorylation (p-FOXO3) as well as deregulation is involved in cancer initiation, progression and drug resistance. Therefore, we proposed that p-FOXO3/FOXO3 ratio change may play important role in the bladder cancer recurrence. Methods: Surgical specimens of cancer tissue were obtained from 75 patients with bladder cancer (30 of non-recurrent and 45 of recurrent). The relative expression levels of p-FOXO3/FOXO3 in cancer tissue were measured by immunohistochemistry (IHC) stain and graded according to stain intensity. The correlation p-FOXO3/FOXO3 with clinicopathological parameters and tumor recurrence was analyzed. Results: For bladder cancer patients with tumor recurrence, higher tumor grade (82% vs 70%, P=0.04) and stage (≥II, 49% vs 33%, P=0.02) in these patients was seen. In IHC study of paired tumor tissues, 39 out of 75 (52%) patients have increased p-FOXO3/FOXO3 ratio and they are closely related to tumor grade (low grade vs high grade =29.4% vs 58.6%, P=0.01) but not related to stage (low stage vs high stage =46.5% vs 59.3%, P=0.26). Regarding to tumor recurrence, the p-FOXO3/FOXO3 ratio is significant higher in recurrent group than non-recurrent group patients (0.78±0.15 vs 1.25±0.11, P=0.03). As comparing the first recurrence and subsequent recurrence group patients, there is no difference in the level of p-FOXO3/FOXO3 ratio (1.25±0.11 vs 1.10±0.09, P=0.25). Interestingly, recurrent tumors in low grade bladder cancer patients have marked increased p-FOXO3/FOXO3 ratio than non-recurrent tumors (0.90±0.22 vs 0.15±0.12, P=0.02). Conclusion: Increased p-FOXO3/FOXO3 ratio has been observed in bladder cancer patients with tumor recurrence and it is closely related to higher tumor grade. Low grade bladder cancer is high risk in recurrence when p-FOXO3/FOXO3 ratio increased. These results implicated that p-FOXO3/FOXO3 ratio can be applied as a useful marker for further treatment decision making and prognostic of tumor recurrence in bladder cancer patients.

Keywords: Bladder cancer, p-FOXO3/FOXO3, clinicopathology, tumor recurrence

Introduction

Bladder cancer is one of the most common malignant tumors and ranks as the 9th leading cause of death worldwide [1, 2]. Besides high morbidity and mortality, bladder cancer is particularly characterized by a high recurrence rate risk [3]. Current prognostic strategies, such as tumor grade, stage, size, and number of foci, have restricted utility for clinicians because they do not specifically exhibit the clinical outcomes of bladder cancer patients [4]. Therefore, the discovery of alternative biomarkers involved in the development and progression of bladder cancer may lead to the identification of new prognostic markers, such as tumor recurrence, disease progression and survival, and therapeutic targets.

The transcription factor forkhead box-O3 (FOXO3, Ser253) is a tumor suppressor gene which regulates cell progression, apoptosis and oxidative stress resistance [5, 6]. Its phosphorylation (p-FOXO3, pSer253) as well as deregulation is involved in cancer initiation, progression and drug resistance. Previous studies have reported that FOXO3 play a vitally important part in the development of many cancers such as breast cancer and prostate cancer [7, 8]. FOXO3 has been shown to inhibit tumor progression

Table 1. Clinicopathological characteristics in non-recurrent and recurrent bladder cancer patients according to age, gender, tumor grade and stag

	Non-recurrent (%) (n=30)	Recurrent (%) (n=45)	*P* value
Age (mean)	70	77	0.19
Gender			
Male	21 (70)	32 (71)	0.92
Female	9 (30)	13 (29)	
Grade			
Low grade	9 (30)	8 (18)	0.04
High grade	21 (70)	37 (82)	
Stage			
I	20 (67)	23 (51)	0.03
II-IV	10 (33)	22 (49)	

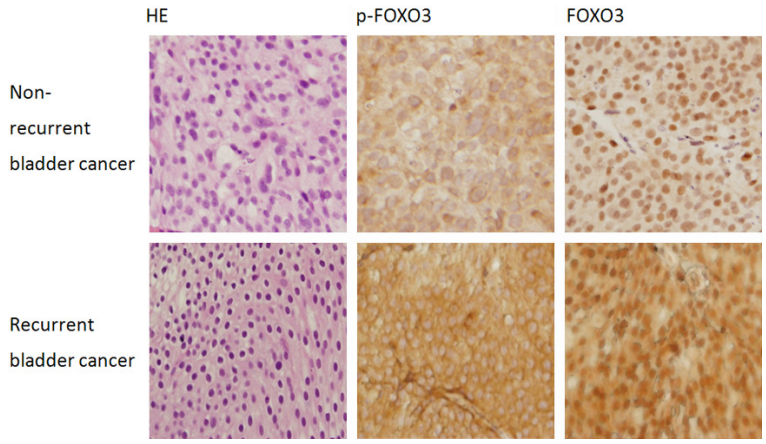

Figure 1. Immunohistochemistry staining of p-FOXO3 and FOXO3 in non-recurrent and recurrent bladder cancer (HE, ×400).

and recurrence in colorectal cancer [9]. Wang et al reported that FOXO3 and FOXO4 expressions may potentially associate with the clinicopathological features and prognosis of bladder cancer [10]. However, correlation between FOXO3 and bladder cancer recurrence was unclear. Hence we investigated the clinicopathologic significance and potential role of FOXO3 in tumor recurrence of bladder cancer in this study.

Materials and methods

Patient population and sample collection

Totally there were 75 patients been enrolled in this study and they had underwent primary cancer surgery (endoscopic resection or radical cystectomy surgery) at Tri-Service General Hospital between years 2009 to 2013. Among them, there were 45 recurrent patients with a control arm of 30 non-recurrent patients. All of these recurrent patients had more than once recurrence during follow up. Blocks of paraffin embedded tissue specimen were confirmed pathologically. The study was approved by the ethics committee of Tri-Service General Hospital (TSGH-IRB-099-05-262). Tumor stage was evaluated according to criteria proposed by AJCC in 2010.

Immunohistochemistry (IHC) stain of tumor specimens

Tissue slides were deparaffinized in xylene, hydrated in an ethanol series, subjected to antigen retrieval by boiling in 10 mM sodium citrate (pH 6.0) for 15 min, and cooled at room temperature (RT) for 20 min. For IHC, after peroxidase blocking (3% hydrogen peroxide in water) for 30 min, slides were washed in water and then blocked in bovine serum albumin (1% BSA) for 15 min. Slides were then incubated with primary rabbit antibody to FOXO3 (1:250, Cell Signaling, Danvers, MA, USA) and p-FOXO3 (1:100, Abcam, San Francisco, USA) for 1 h at RT, subjected to a TBST (1 M Tris-HCL, 5 M NaCl, 0.1% Tween-20) wash series, incubated with goat-anti-rabbit secondary antibody (Novolink, Leica, Germany) for 30 min at RT and subjected to a second TBST wash series. Signal was detected using a DAB liquid chromogen substrate kit (Thermo, USA). Slides are then counterstained with hematoxylin, rinsed in water, and air-dried. The intensity of stain was observed and graded to 0, 1, 2, and 3 categories and the ratio of p-FOXO3 and FOXO3 was measured and expressed as >1, =1, or <1.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Categorical variables were presented as count or percentage. Student's t-test was used to assess continuous variables, with χ^2-test employed for categorical variables. All

Table 2. Correlation between p-FOXO3/FOXO3 ratio and clinicopathological characteristics of bladder cancer patients

	Case No. (%)	p-FOXO3/FOXO3 ratio No. (%)		*P* value
		Increased (>1.0)	Decreased (≤1.0)	
Gender				
Male	53 (70.7)	26 (49.1)	27 (50.9)	0.44
Female	22 (29.3)	13 (59.1)	9 (40.9)	
Tumor Grade				
Low grade	17 (22.7)	5 (29.4)	13 (70.6)	0.02
High grade	58 (77.3)	34 (58.6)	23 (41.4)	
Tumor Stage				
I	43 (57.3)	20 (46.5)	23 (53.5)	0.26
II-IV	32 (42.7)	19 (59.3)	13 (40.7)	

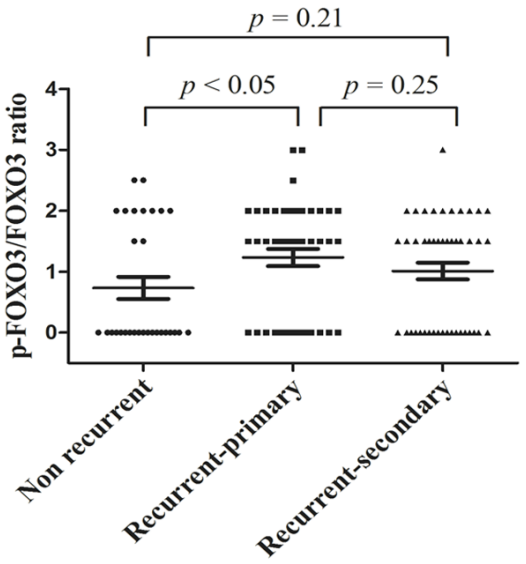

Figure 2. The distribution of p-FOXO3/FOXO3 ratio among patients with non-recurrent (n=30), primary recurrent (n=45) and their subsequent recurrent bladder cancer tumors. The p-FOXO3/FOXO3 ratio is significant higher in recurrent group than non-recurrent group patients (0.78±0.15 vs 1.25±0.11, P=0.03). As comparing the first recurrence and subsequent recurrence group patients, there is no difference in the level of p-FOXO3/FOXO3 ratio (1.25±0.11 vs 1.10±0.09, P=0.25).

the statistical analyses were performed using the SPSS 16.0 and Excel 2007. P<0.05 was considered statistically significant.

Results

Patient characteristics

As shown in **Table 1**, there are no significant differences between these two group patients in age, gender, tumor grade and stage distribution. Most patients were aged people and male (53/75; 70.7%). Most tumors were high grade (58/75, 77.3%) and low stage (43/75, 57.3%). For bladder cancer patients with tumor recurrence, higher tumor grade (82% vs 70%, P=0.04) and higher stage (≥II, 49% vs 33%, P=0.02) in these patients was seen.

p-FOXO3/FOXO3 ratio and clinicopathological characteristics

In IHC study of paired bladder tumor tissues for the expression of p-FOXO3 and FOXO3, 39 out of 75 (52%) patients have increased p-FOXO3/FOXO3 ratio and they are closely related to tumor grade (high grade vs low grade =58.6% vs 29.4%, P=0.01) but not related to stage (55.8% vs 59.4%, P=0.26) (**Figure 1** and **Table 2**). No gender difference was seen in the expression of p-FOXO3 and FOXO3 in bladder cancer.

p-FOXO3/FOXO3 ratio and bladder cancer recurrence

As shown in **Figure 2**, the p-FOXO3/FOXO3 ratio in the first recurrent bladder cancer tumors is significant higher than non-recurrent bladder cancer tumors (1.25±0.11 vs 0.78±0.15, P=0.03). As comparing the first recurrence and subsequent recurrence episode in recurrent group patients, there is no difference in the level of p-FOXO3/FOXO3 ratio (1.25±0.11 vs 1.10±0.09, P=0.25) although they are all increased. When stratified non-recurrent and recurrent patients according to tumor grade and p-FOXO3/FOXO3 ratio, we found that low grade tumors with decreased p-FOXO3/FOXO3 ratio have lowest recurrent rate than low grade tumor with increased p-FOXO3/FOXO3 ratio and high grade tumors nevertheless the variation of p-FOXO3/FOXO3 ratio (**Figure 3**).

Discussion

The FOXO transcription factors have been reported to play pivotal roles in tumorigenesis and drug resistance. The mechanisms underlying the tumor suppression function of FOXOs in human cancers remain largely unknown. The role of FOXO3 have been fully evaluated in vari-

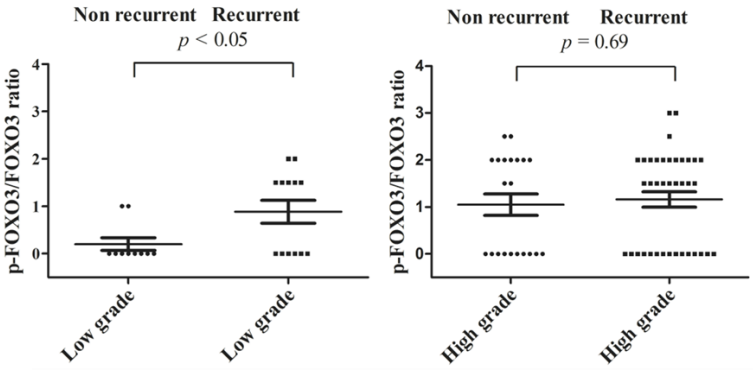

Figure 3. The p-FOXO3/FOXO3 ratio distribution of non-recurrent and recurrent tumors in low grade (n=18) and high grade bladder cancer (n=57). Recurrent tumors in low grade bladder cancer patients have marked increased p-FOXO3/FOXO3 ratio than non-recurrent tumors (0.90±0.22 vs 0.15±0.12, P=0.02) while there is no significant difference among high grade tumors.

ous cancers on prognosis (5-yr survival rate, disease free survival), metastasis and disease progression, including gastric cancer [11, 12], colon cancer [13], breast cancer [14], ovarian cancer [15], lung cancer [16], and renal cancer [17]. All of these studies confirmed the FOXO3 as an important molecular marker for prognosis, invasiveness, and progression of cancers. These results also imply that FOXO3 is closely related to carcinogenesis and drug resistance in addition to tumor behavior.

In 2010, Shiota et al first reported that FOXO3a expression decreased in invasive bladder cancer and patients with low FOXO3a expression had poor disease-free survival, cancer-specific survival, and overall survival [18]. FOXO3a knockdown in urothelial cancer cells increased cellular motility. FOXO3a negatively regulated Twist1 and Y-box-binding protein 1, and positivelyregulated E-cadherin. They suggest that FOXO3a could act as an independent prognostic factor in bladder cancer and could represent a promising molecular target for cancer therapeutics. Later on, Wang et al reported that FOXO3 and FOXO4 expressions may potentially associate with the clinicopathological features and prognosis of bladder cancer [10].

Cho et al discovered tumor suppressor FOXO3 as the novel regulator of RRM2B and overexpression of RRM2B and/or FOXO3 inhibited the proliferation of cancer cells [19]. RRM2B was identified as a p53-inducible ribonucleotide reductase (RR) subunit that involves in various critical cellular mechanisms such as cell cycle regulation, DNA repair and replication, and mitochondrial homeostasis, etc. They also demonstrated a strong correlation between the co-expression of FOXO3 plus RRM2B and increased disease survival and reduced recurrence or metastasis in lung cancer patients. Their results suggest that FOXO3 and RRM2B could be used as predictive biomarkers for cancer progression.

The transcription factor FOXO3 is a well-established tumor suppressor whose activity, stability, and localization are regulated by phosphorylation and acetylation [20]. Previous studies emphasized the role of FOXO3 alone in bladder cancer prognosis [10, 18], in this study we further evaluated with variation of p-FOXO3 and FOXO-3 and its correlation with bladder cancer recurrence. We found that 52% of bladder cancer patients have increased p-FOXO3/FOXO3 ratio and they are closely related to tumor grade but not related to stage. Furthermore, in addition to tumor grade and stage, significantly increased p-FOXO3/FOXO3 ratio was seen in recurrent bladder cancers than non-recurrent bladder cancers. No difference of p-FOXO3/FOXO3 ratio was seen between first recurrent and subsequent recurrent bladder tumors, it indicates that the p-FOXO3/FOXO3 ratio change within tumor cells is constant after tumor recurrence. We first noted that in low grade bladder cancers with increased p-FOXO3/FOXO3 ratio have higher recurrence rate than tumors with lower level of p-FOXO3/FOXO3 ratio. It indicates that low grade bladder cancers can be stratified into high or low risk of recurrence clinically according to the ratio of p-FOXO3/FOXO3. These results still need further verified in the future since our case number enrolled in this preliminary study is limited.

In summary, this study first demonstrated that tumor recurrence in bladder cancer patients is closely related to increased p-FOXO3/FOXO3 ratio in addition to tumor grade and stage. Low grade bladder cancer is high risk in recurrence when p-FOXO3/FOXO3 ratio increased. These findings suggest that p-FOXO3/FOXO3 ratio can be applied as a useful marker for further treatment decision making and prognosis in facing bladder cancer patients.

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Disclosure of conflict of interest

None.

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