Forkhead box O1 as an indicator of prognosis is inactivated in urothelial carcinoma of the upper urinary tract

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Abstract. The transcription factor forkhead box O1 (FOXO1) can be inactivated via its phosphorylation, resulting in suppression of apoptosis. Using immunohistochemistry, the expression of a phosphorylated form of FOXO1 was assessed in upper urinary tract urothelial carcinoma (UUTUC) specimens. Overall, phospho-FOXO1 (p-FOXO1) was immunoreactive in all 99 UUTUC specimens [12 (12.1%) weak (1+), 46 (46.5%) moderate (2+) and 41 (41.4%) strong (3+)], which was significantly (P=0.018) increased, compared with benign urothelium specimens [77/82 (93.9%): 18 (22.0%) 1+, 41 (50.0%) 2+ and 18 (22.0%) 3+]. Muscle invasion (P=0.031) and lymphovascular invasion (P=0.025) were observed more frequently in p-FOXO1(2+/3+) tumor samples compared with p-FOXO1(1+) tumor samples. No statistically significant associations between p-FOXO1 expression and tumor grade or presence of concurrent carcinoma *in situ*, hydronephrosis or lymph node metastasis were observed. Furthermore, the levels of p-FOXO1 and estrogen receptor-β expression were significantly (P<0.05) correlated in UUTUC samples [correlation coefficient (CC)=0.244], particularly in tumor samples from male patients (CC=0.330). Additionally, patients with p-FOXO1(3+) tumors had a significantly increased risk of cancer-specific mortality (P=0.043), compared with those with p-FOXO1(1+/2+) tumors. Multivariate analysis further demonstrated a notable, albeit not significant, association between p-FOXO1 expression and cancer-specific survival (hazard ratio=2.204; P=0.053). These findings indicate that FOXO1 is inactivated in UUTUC specimens and p-FOXO1 overexpression may serve as a predictor of poor patient outcomes.

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Introduction

Upper urinary tract cancer is a relatively rare, but frequently aggressive, malignancy, which is primarily derived from urothelial cells (1,2). Owing to the lower incidence of upper urinary tract urothelial carcinoma (UUTUC), compared with bladder cancer, there is limited knowledge regarding specific molecular or biological changes in UUTUC. Notably, no established prognostic biomarkers that are beneficial for decision-making of the management of UUTUC are available (3-5).

Forkhead box O1 (FOXO1), a forkhead transcription factor, is known to be inactivated by phosphorylation through the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, resulting in suppression of apoptosis and regulation of the cell cycle, as well as control of cell invasion, in prostate and colon cancer lines (6,7). In bladder cancer cells, microRNA-96 (8) and a derivative of a Chinese herb isorhapontigenin (9) have been demonstrated to mediate apoptosis and invasion, respectively, via targeting FOXO1. Additionally, potential cross-talk between FOXO1 and nuclear receptors, particularly sex hormone receptors, including androgen receptor (AR) and estrogen receptor-β (ERβ), has been identified in prostate cancer cells (10,11). Furthermore, an increasing amount of preclinical evidence has indicated a critical role for AR and ERβ signals in the development and progression of urothelial cancer (12-14).

Previous immunohistochemical studies in bladder cancer specimens have indicated that loss of or decreased expression of FOXO1, as a tumor suppressor, is associated with poorer patient outcomes (15-17). By contrast, the expression status of phosphorylated forms of FOXO1 and its prognostic value in urothelial cancer, as well as the functions of FOXO1 in UUTUC, remain poorly understood. The present study aimed to determine the status of phospho-FOXO1 (p-FOXO1) expression in UUTUC and its associations with clinicopathological characteristics.

Materials and methods

UUTUC tissue microarray (TMA). A UUTUC TMA was constructed with formalin-fixed paraffin embedded specimens [(tumor samples (n=99)] and paired normal-appearing urothelial tissues (n=82) from patients [60 men and 39 women with a mean/median age of 70.0/71 years (range: 48-87 years)] who underwent radical nephroureterectomy between March 1997 and September 2011, as described previously (18). All sections were reviewed by a urologic pathologist (at The Johns Hopkins Hospital; 18) for confirmation of the original diagnosis of urothelial carcinoma and tumor grade/stage of each case according to the World Health Organization histological classification (2004)/TNM classification (American Joint Committee on Cancer 7th Edition) of tumors of the urinary tract/renal pelvis and ureter, respectively (19). Appropriate approval from the institutional review board at Osaka General Medical Center was obtained prior to construction and use of the TMA. Clinicopathological characteristics of these patients (see Table I) were obtained from medical records and follow-up data. No patients had received neoadjuvant treatment or other anticancer therapies, including irradiation, prior to nephroureterectomy.

Immunostaining. Immunohistochemistry was performed, as described previously (20). Briefly, the 5 μm sections from the TMA were stained, using a primary antibody against p-FOXO1 (Ser^{256}; cat no SAB4300094; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; 1:200; 4˚C overnight) and a broad-spectrum secondary antibody (cat. no. 959643; Histostain-SP IHC kit, DAB; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA; 10 min at room temperature plus 10 min with enzyme conjugated solution at room temperature). All stains were manually quantified by two pathologists who were blinded to the identity of the samples. The German immunoreactive scores calculated by multiplying the percentage of immunoreactive cells (0%, 0; 1-10%, 1; 11-50%, 2; 51-80%, 3; and 81-100%, 4) by staining intensity (negative, 0; weak, 1; moderate, 2; and strong, 3) were grouped as negative (0; score 0-1), weakly positive (1+; score 2-4), moderately positive (2+; score 6-8) or strongly positive (3+; score 9-12).

Statistical analyses. Data are presented as mean ± standard deviation. Fisher's exact test and Student's t-test were used to evaluate the association between categorized variables and those with a continuous distribution, respectively. Correlations between variables were determined by the Pearson's correlation coefficient (CC). The rates of recurrence-free survival, progression-free survival (PFS) and cancer-specific survival (CSS) were calculated by the Kaplan-Meier method, and comparisons were analyzed using the log-rank test. Disease progression was defined as the development of non-bladder lesions, including recurrence at the nephroureterectomy site and lymph node or visceral metastasis. Metachronous or synchronous recurrence in the lower urinary tract was not considered as tumor progression. Cox proportional hazards model was used to determine the statistical significance of prognostic indicators in a multivariate setting. These statistical analyses were performed using SPSS (version 22; IBM Corp., Armonk, NY, USA) or GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of p-FOXO1 in tumor and corresponding non-neoplastic tissues. In the present study, 99 UUTUC specimens and 82 matched normal-appearing urothelial tissues were immunohistochemically stained for an inactivated form of FOXO1 (p-FOXO1). Positive signals for p-FOXO1 were primarily detected in the nuclei of non-neoplastic (Fig. 1A) and neoplastic (Fig. 1B) urothelial cells. p-FOXO1 was expressed in 77/82 (93.9%) benign urothelial tissues [18 (22.0%) 1+, 41 (50.0%) 2+ and 18 (22.0%) 3+] and all 99 (100%) urothelial neoplasms [12 (12.1%) 1+, 46 (46.5%) 2+ and 41 (41.4%) 3+]. Thus, the levels of p-FOXO1 expression were significantly increased in tumor samples, compared with benign tissues (0 vs. 1+/2+/3+, P=0.018; 0/1+ vs. 2+/3+, P=0.008; and 0/1+/2+ vs. 3+, P=0.007).

Association of p-FOXO1 expression with clinicopathological features of UUTUC samples. Table I displays the levels of p-FOXO1 expression in UUTUC samples on the basis of their clinicopathological characteristics. The expression of p-FOXO1 was significantly (1+ vs. 2+/3+: P=0.031) increased in muscle-invasive (≥pT2) tumor samples, compared with non-muscle-invasive (≤pT1) tumor samples, whereas it was not statistically different between low-grade and high-grade carcinoma samples. Additionally, the rate of lymphovascular invasion was significantly (P=0.025) increased in p-FOXO1(2+/3+) tumor samples [39/87 (44.8%)], compared with p-FOXO1(1+) tumor samples [1/12 (8.3%)]. There were no significant associations between the levels of p-FOXO1 expression and other characteristics, including patient age or sex, tumor laterality or site, and presence of concomitant carcinoma *in situ*, hydronephrosis or lymph node metastasis.

Subsequently, the associations between the expression of p-FOXO1 and steroid hormone receptors including AR, ERα, ERβ, glucocorticoid receptor (GR) and progesterone receptor (PR), were assessed. In the same cohort of 99 patients with UUTUC, it was demonstrated previously that AR, ERα, ERβ, GR and PR were positive in 20 (20.2%), 18 (18.2%), 62 (62.6%), 62 (62.6%) and 16 (16.2%) UUTUC samples, respectively (20). A significant ($P<0.05$) weak positive (CC=0.2-0.4) correlation between p-FOXO1 and ERβ expression (CC=0.244; P=0.015), particularly in tumor samples from male patients (CC=0.330; P=0.010), was observed (Table II). Specifically, of 58 p-FOXO1(1+/2+) vs. 41 p-FOXO1(3+) tumor samples, 29 (50.0%) vs. 33 (80.5%) were immunoreactive for ERβ (P=0.003), respectively. Similarly, of 34 p-FOXO1(1+/2+) vs. 26 p-FOXO1(3+) tumor samples from male patients, 14 (41.2%) vs. 21 (80.8%) were immunoreactive for ERβ (P=0.003), respectively. However, p-FOXO1 levels were not significantly associated with the positivity of AR, ERα, GR or PR in all 99, 60 male or 39 female tumor samples.

Association of p-FOXO1 expression with patient outcomes. Kaplan-Meier analysis coupled with the log-rank test was performed to assess the prognostic values of p-FOXO1 expression in 95 UUTUC samples with no distant metastasis at the time of nephroureterectomy. There were no significant associations between p-FOXO1 levels and tumor recurrence in the bladder (1+ vs. 2+ vs. 3+, P=0.705; 1+ vs. 2+/3+, P=0.406; 1+/2+ vs. 3+, P=0.852). However, the increased expression

Table I. Association between p-FOXO1 expression and clinicopathological profile of the patients.

		p-FOXO1 expression			P-value	
Parameters	n	1+ (%)	2+ (%)	3+ (%)	1+ vs. 2+/3+	1+/2+ vs. 3+
Age (mean ± SD; years)	99	67.4±10.6	70.8±8.4	69.8±9.2	0.384	0.869
Sex					0.532	0.680
Male	60	6 (10.0)	28 (46.7)	26 (43.3)		
Female	39	6 (15.4)	18 (46.2)	15 (38.5)		
Laterality					0.120	0.220
Right	43	8 (18.6)	14 (32.6)	21 (48.8)		
Left	56	4 (7.1)	32 (57.1)	20 (35.7)		
Tumor site					0.763[c]	0.834[c]
Renal pelvis	45	5 (11.1)	21 (46.7)	19 (42.2)		
Ureter	50	7 (14.0)	24 (48.0)	19 (38.0)		
Both	4	0 (0)	1 (25.0)	3 (75.0)		
Tumor grade[a]					0.686	1.000
Low-grade	15	1 (6.7)	8 (53.3)	6 (40.0)		
High-grade	84	11 (13.1)	38 (45.2)	35 (41.7)		
Pathological stage[b]					0.031[d]	0.207[d]
pTa	19	3 (15.8)	9 (47.4)	7 (36.8)		
pT1	18	5 (27.8)	8 (44.4)	5 (27.8)		
NMI (pTa + pT1)	37	8 (21.6)	17 (45.9)	12 (32.4)		
pT2	8	2 (25.0)	4 (50.0)	2 (25.0)		
pT3	48	2 (4.2)	24 (50.0)	22 (45.8)		
pT4	6	0 (0)	1 (16.7)	5 (83.3)		
MI (pT2 + pT3 + pT4)	62	4 (6.5)	29 (46.8)	29 (46.8)		
Concurrent CIS					1.000	1.000
No	86	10 (11.6)	40 (46.5)	36 (41.9)		
Yes	13	2 (15.4)	6 (46.2)	5 (38.5)		
Hydronephrosis					1.000[e]	0.604[e]
No	61	8 (13.1)	28 (45.9)	25 (41.0)		
Yes	20	2 (10.0)	8 (40.0)	10 (50.0)		
Unknown	18	2 (11.1)	10 (55.6)	6 (33.3)		
Lymphovascular invasion					0.025	0.096
No	59	11 (18.6)	28 (47.5)	20 (33.9)		
Yes	40	1 (2.5)	18 (45.0)	21 (52.5)		
Lymph node involvement[b]					1.000[f]	0.756[f]
pN0	84	11 (13.1)	38 (45.2)	35 (41.7)		
pN1-3	12	1 (8.3)	7 (58.3)	4 (33.3)		
pNx	3	0 (0)	1 (33.3)	2 (66.7)		

[a]World Health Organization histological classification (2004) (ref. 19); [b]TNM classification (American Joint Committee on Cancer 7th Edition) (ref. 19); [c]Renal pelvis vs. ureter; [d]NMI vs. MI; [e]No vs. yes; [f]pN0 vs. pN1-3. NMI, non-muscle-invasive; MI, muscle-invasive; CIS, carcinoma *in situ*; p-FOXO1, phospho-forkhead box O1; SD, standard deviation; 1+, weakly positive; 2+, moderately positive; 3+, strongly positive.

of p-FOXO1 was insignificantly and significantly associated with decreased PFS [(1+ vs. 2+ vs. 3+, P=0.134 (Fig. 2A); 1+ vs. 2+/3+, P=0.099 (Fig. 2B); 1+/2+ vs. 3+, P=0.341) and CSS (1+ vs. 2+ vs. 3+, P=0.045 (Fig. 2C); 1+ vs. 2+/3+, P=0.249; 1+/2+ vs. 3+, P=0.043 (Fig. 2D)], respectively.

To determine whether the status of p-FOXO1 expression was an independent indicator of prognosis in the 95 patients with UUTUC, multivariate analysis, including the factors demonstrating statistical significance in univariate analysis, was performed with the Cox model (Table III). Although tumor grade, pT stage and pN stage were not significantly associated with CSS, lymphovascular invasion was identified to be an independent factor for CSS [hazard ratio (HR)=3.222; P=0.028]. Additionally, there was a notable, albeit not significant, association between p-FOXO1 expression and CSS (HR=2.204; P=0.053).

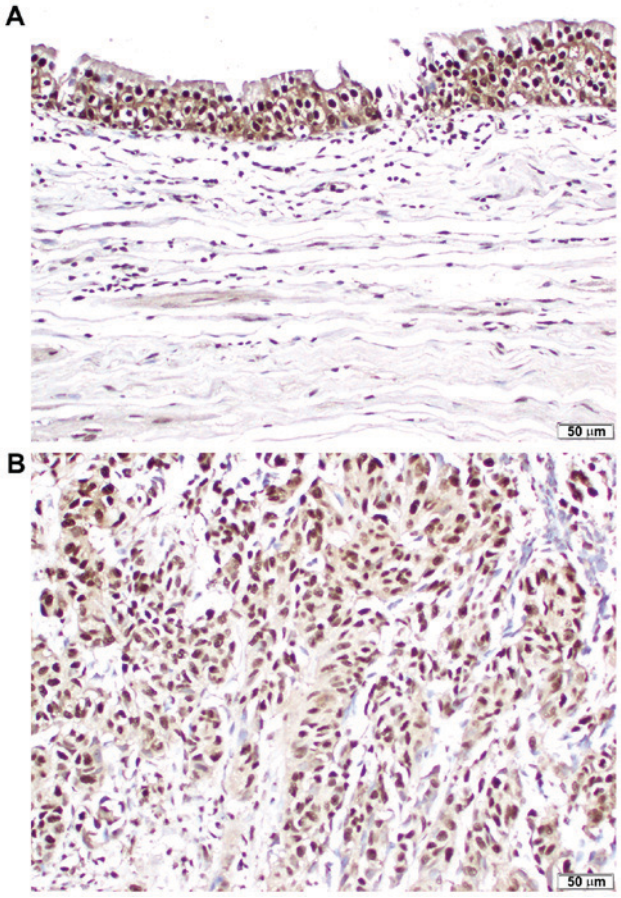

Figure 1. Immunohistochemistry of p-FOXO1 in (A) normal urothelium and (B) urothelial tumor specimens. A semi-quantitative analysis of p-FOXO1 expression was performed by employing a combination of staining intensity and distribution. Original magnification, x200. p-FOXO1, phospho-forkhead box O1.

Discussion

There is limited knowledge regarding the function of the potential tumor suppressor FOXO1 in urothelial carcinogenesis and tumor growth. Furthermore, to the best of our knowledge, the status of FOXO1 or p-FOXO1 expression in UUTUC has not previously been investigated. Using immunohistochemistry, the levels of p-FOXO1 expression were compared in UUTUC samples and corresponding adjacent normal-appearing tissues in the upper urinary tract and it was demonstrated that they were significantly increased in tumor cells, compared with the non-neoplastic urothelial cells. Consistent with the present data, a recent study demonstrated downregulation of FOXO1 expression in bladder cancer, compared with non-cancerous bladder mucosa (17). These observations may indicate that FOXO1 contributes to the prevention of urothelial tumorigenesis. Relatively high levels of p-FOXO1 expression in normal-appearing urothelial tissues in the TMA used in the present study may be due to the cancer field effect (21) that potentially affected the immunoreactivity.

FOXO1 has also been implicated in the regulation of cell proliferation, apoptosis and cell cycle control, as well as cell migration and invasion, via its phosphorylation/inactivation through the PI3K/Akt signaling pathway (6,7). In the present study, p-FOXO1 overexpression was identified to be associated with muscle invasion and lymphovascular invasion in UUTUC. Univariate and multivariate analyses further demonstrated that p-FOXO1 overexpression was significantly and insignificantly, respectively, associated with cancer-specific mortality in patients with UUTUC. Additionally, a few immunohistochemical studies in bladder cancer tissue samples have indicated the prognostic significance of FOXO1 expression (15-17). Thus, FOXO1 activity is indicated to predict the prognosis of UUTUC. However, although FOXO1 expression in superficial bladder tumor samples was demonstrated to predict the risk of their recurrence (15), the present study did not indicate a significant association between p-FOXO1 overexpression in UUTUC samples and its recurrence in the bladder.

The functional interplay between FOXO1 and steroid hormone receptors, particularly AR and ERβ, has been demonstrated previously. Specifically, androgen and estrogen were able to reduce the expression or activity of FOXO1 in the presence of AR and ERβ, respectively, in prostate cancer cells (10,11). Estrogen-mediated ER (ERα, ERβ or both) signals were also demonstrated to induce phosphorylation of FOXO1 in breast cancer cells (22). In bladder cancer cell lines, it was identified that androgens and estrogens could inactivate FOXO1 via the AR and ERβ pathways, respectively (Ide *et al*, unpublished data). In accordance with these observations, the present study indicated that p-FOXO1 expression was significantly correlated with ERβ expression in UUTUC samples. However, the levels of p-FOXO1 expression were not significantly correlated with the expression of other steroid hormone receptors, including AR, ERα, GR and PR.

Using the identical UUTUC TMA, we previously determined the expression status of various transcription factors (20,23-26). Notably, the positive rates of 6 transcription factors, including AR (20), ERβ (20), GATA-binding protein 3 (GATA3) (23), zinc finger with KRAB and SCAN domains 3 (ZKSCAN3) (24), nuclear factor of activated T-cells 1 (NFATc1) (25) and a phosphorylated form of ELK1 (p-ELK1) (26), were significantly ($P<0.05$; GATA3, ZKSCAN3) or insignificantly ($0.05\leq P<0.1$; AR, ERβ, NFATc1, p-ELK1) different between renal pelvic and ureteral tumor samples, although the underlying reasons remain unclear. However, similar to other transcription factors previously examined, including ERα (20), GR (20) and PR (20), no significant change ($P\geq0.1$) in the levels of p-FOXO1 expression at different sites of UUTUC was identified in the present study.

The function of the potential tumor suppressor FOXO1 in the development and progression of UUTUC remains poorly understood. In the present study, the expression status of p-FOXO1 in UUTUC specimens and its prognostic significance were immunohistochemically determined. The levels of p-FOXO1 expression were compared in tumor samples and adjacent normal tissues in the upper urinary tract, and it was identified that p-FOXO1 expression was significantly upregulated in UUTUC, compared with non-neoplastic urothelium. A recent study also demonstrated downregulation of FOXO1 expression in bladder cancer, compared with non-cancerous bladder mucosa (17). These results indicate that FOXO1 may contribute to the prevention of urothelial tumorigenesis.

In conclusion, a significant increase in the expression of p-FOXO1 in UUTUC samples, compared with corresponding normal-appearing urothelial tissues, was demonstrated, implying the involvement of FOXO1, as a tumor suppressor,

Table II. Correlation between p-FOXO1 and AR/ERα/ERβ/GR/PR expression.

		AR		ERα		ERβ		GR		PR	
Patients	n	CC	P-value	CC	P-value	CC	P-value	CC	P-value	CC	P-value
All cases	99	0.155	0.125	-0.011	0.917	0.244	0.015	0.088	0.384	-0.069	0.497
Males	60	0.213	0.103	0.184	0.160	0.330	0.010	0.036	0.783	0.085	0.516
Females	39	0.009	0.955	-0.259	0.111	0.141	0.392	0.143	0.386	-0.195	0.235

p-FOXO1, phospho-forkhead box O1; AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; PR, progesterone receptor.

Table III. Univariate and multivariate analysis of cancer-specific survival in 95 patients with upper urinary tract urothelial carcinoma.

	Univariate			Multivariate		
Parameter	HR	95% CI	P-value	HR	95% CI	P-value
Tumor grade	6.411	0.868-47.372	0.036	4.770	0.626-36.331	0.131
pT stage[a]	3.416	1.598-7.305	0.002	2.047	0.712-5.883	0.184
LVI	6.712	2.827-15.934	<0.001	3.222	1.132-9.168	0.028
pN stage	4.379	1.762-10.884	0.001	2.348	0.815-6.761	0.114
p-FOXO1[b]	2.262	1.028-4.975	0.043	2.204	0.989-4.910	0.053

[a]pTa-2 vs. pT3-4; [b]1+/2+ vs. 3+. LVI, lymphovascular invasion; HR, hazard ratio; CI, confidence interval; p-FOXO1, phospho-forkhead box O1.

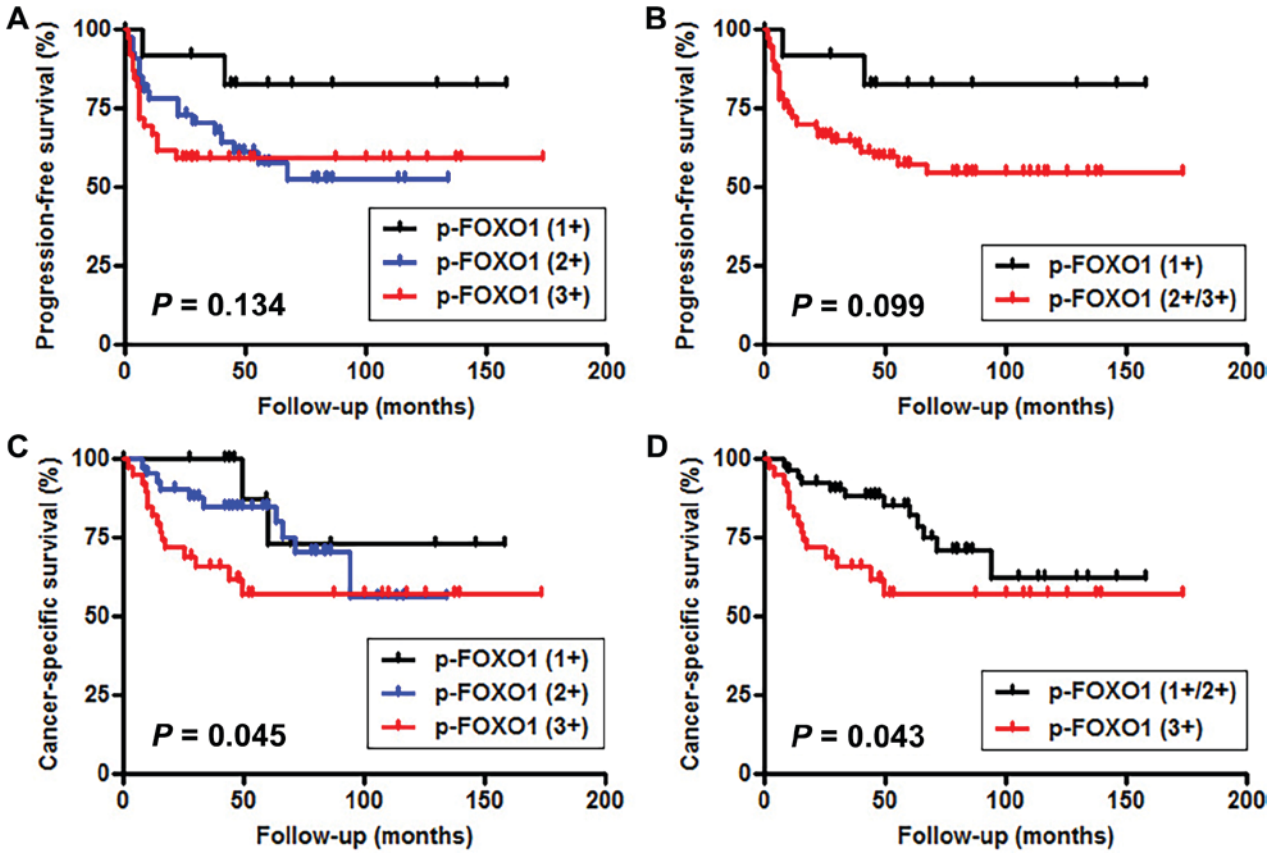

Figure 2. Kaplan-Meier curves for PFS in 95 patients without metastatic disease, according to the levels of p-FOXO1 expression (A) 1+ vs. 2+ vs. 3+; (B) 1+ vs. 2+/3+ and for CSS in 95 patients without metastatic disease, according to the levels of p-FOXO1 expression (C) 1+ vs. 2+ vs. 3+; (D) 1+/2+ vs. 3+. p-FOXO1, phospho-forkhead box O1; PFS, progression-free survival; CSS, cancer-specific survival.

in the outgrowth of UUTUC. The results of the present study further indicate that p-FOXO1 overexpression serves as a predictor of poor prognosis in patients with UUTUC.

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Availability of data and materials

All data used or analyzed used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HI and HM conceived and designed the study. HI, GJ and SI performed the experiments. HI, GJ, TM, KF, SY, HF and NN analyzed the data. KF, SY, HF and NN contributed reagents, materials and analysis tools. HI drafted the manuscript and HM edited it. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present research study was conducted with the approval from the Institutional Review Board at Osaka General Medical Center (Osaka, Japan; IRB no. 25-2014).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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