

Low PTEN expression and overexpression of phosphorylated Akt^{Ser473} and Akt^{Thr308} are associated with poor overall survival in upper tract urothelial carcinoma

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Abstract. The PI3K/Akt signaling pathway serves an essential role in various cellular processes, including cell growth, survival, cell motility, angiogenesis and cell metabolism. Loss of PTEN expression and hyperactivation of Akt can result in tumorigenesis. Previous studies observed expression of the Akt protein and absence of the PTEN protein in bladder cancer and non-small cell lung carcinoma tissues. The aim of the present study was to evaluate the expression status and prognostic value of PTEN and the PI3K/Akt signaling pathway in Taiwanese patients with upper tract urothelial carcinoma (UTUC). Archival formalin-fixed, paraffin-embedded (FFPE) tissues from 65 UTUC cases were stained via immunohistochemistry for PTEN, phosphorylated (p)Akt serine (Ser)⁴⁷³ and pAkt threonine (Thr)³⁰⁸. The expression levels of each protein were significantly correlated with clinicopathological parameters. PTEN, pAkt^{Ser473} and pAkt^{Thr308} protein expression levels were higher in adjacent normal tissues compared with those in tumor tissues. Cytoplasmic PTEN protein expression levels were lower in high-stage tumors compared

with those in low-stage tumors, and nuclear and cytoplasmic pAkt^{Thr308} protein expression levels were higher in high-grade tumors compared with those in low-grade tumors. Univariate analysis showed that high pathological tumor stage (pT2-4) [P=0.01; hazard ratio (HR)=3.40; 95% confidence interval (CI), 1.34-8.60], metastatic status (P=0.003; HR=3.55, 95% CI, 1.55-8.11), low cytoplasmic PTEN protein expression levels (P=0.016; HR=3.14; 95% CI, 1.24-7.95) and high cytoplasmic pAkt^{Ser473} protein expression levels (P=0.019, HR=2.71, 95% CI, 1.18-6.21) were predictive of poor overall survival. However, only metastatic status (P=0.031; HR=2.73; 95% CI, 1.10-6.78), low cytoplasmic PTEN protein expression levels (P=0.017; HR=3.29; 95% CI, 1.24-8.73) and high cytoplasmic pAkt^{Ser473} protein expression levels (P=0.027; HR=2.64; 95% CI, 1.12-6.23) remained significant in the multivariate analysis. Kaplan-Meier survival analysis showed that high T stage, metastasis, low expression levels of cytoplasmic PTEN protein and high expression levels of cytoplasmic pAkt^{Ser473} protein were significantly associated with poor survival (P=0.006, 0.001, 0.011 and 0.014, respectively). Co-expression of PTEN^{low}/pAkt^{Ser473/high} and pAkt^{Thr308/high} phenotypes was associated with a less favorable overall survival (P=0.001). Overall, the present findings demonstrated that low expression levels of PTEN and high expression levels of pAkt^{Ser473} and pAkt^{Thr308} were predictors for poor overall survival in patients with UTUC.

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Abbreviations: UCs, urothelial carcinomas; UTUC, upper tract urothelial carcinoma; BC, bladder cancer; PIP3, phosphatidyl-inositol (3,4,5)-triphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence *in situ* hybridization

Key words: PTEN, pAkt^{Ser473}, pAkt^{Thr308}, immunohistochemistry, multivariate analysis, overall survival, Kaplan-Meier analysis, upper tract urothelial carcinoma

Introduction

Malignant tumor cells derived from the urinary epithelium are called urothelial carcinomas (UCs), and these constitute the fourth most common cancer in the world (1). Bladder cancer (BC) is the most common type of UC, and accounts for 90-95% of the total UC cases (2). In Western countries, the prevalence of upper tract urothelial carcinomas (UTUCs) is notably less compared with that of BC, which accounts for only 5-10% of all UC cases, and the incidence of UTUC in the renal pelvis is ~2-3-fold more common than that in the ureter,

and the male-to-female ratio is ~2-3:1 (3). However, in Taiwan, UTUCs account for 30% of all UC cases, and the incidence of UTUC in the renal pelvis is similar to that in the ureter with a ratio of ~1.1:0.9 (4). In addition, there are more female patients with UTUC than males (5). The high recurrence rate, high progression potential and frequent distant metastasis are the main reasons why UC has poor clinical outcomes even when diagnosed at an early stage. In a clinical setting, previous studies have shown that tumor (T) stage, tumor grade, tumor size and lymph node metastasis are important prognostic predictors for UTUC (6,7). The present study aimed to identify the biomarkers of advanced UTUC based on pathological features from human tissue samples.

PTEN is a dual protein/lipid phosphatase that dephosphorylates phosphatidyl-inositol (3,4,5)-triphosphate (PIP3) into phosphatidylinositol 4,5-bisphosphate (PIP2). Deletion, mutation or silencing due to high levels of promoter methylation causes loss of PTEN activity in a number of primary and metastatic cancers (8,9). Moreover, loss of PTEN is associated with an aggressive tumor phenotype and poor clinical outcomes in UC (10). Concordantly, PTEN is also a negative regulator of the PI3K/Akt/mTOR signaling pathway (11) (Fig. 1). Akt is recruited to the cell membrane by PIP3, and Phosphoinositide-dependent kinase-1 (PDK1) phosphorylates Akt on threonine (Thr)308 (12). The mammalian target of rapamycin complex 2 (mTORC2) complex then phosphorylates Akt on serine (Ser)473 via a positive feedback loop (13), leading to full activation of Akt. Phosphorylated Akt activates mTORC1 to regulate the phosphorylation of the S6 protein and the initial translational factor in eukaryotes, eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (14). Activated Akt phosphorylates several downstream effectors that regulate a variety of essential processes such as cell growth, cell metabolism, cell survival and protein synthesis (15). Although the phosphorylation of both Thr308 and Ser473 is thought to be mandatory for complete activation of the Akt pathway, there are still discrepancies about the association of this with clinical outcomes. Gallay *et al* (16) demonstrated that the levels of phosphorylation on Thr308, instead of on Ser473, were significantly associated with poor clinical outcomes, including overall survival, event-free survival and relapse-free survival, in acute myeloid leukemia. By contrast, Freudlsperger *et al* (17) showed that the relative levels of phosphorylated (p)Akt on Ser473 were associated with overall survival and progression-free survival in patients with advanced head and neck squamous cell carcinoma, but the relative levels of pAkt on Thr308 were not correlated with patient outcomes.

Activation of PI3K and Akt is reported to induce ovarian (18), breast (18,19), esophageal (20) and pancreatic cancer (21), among other (9,22). The PI3K/Akt/mTOR signaling pathway serves an essential role in various cellular processes, including cell growth, cell motility, cell survival, angiogenesis and cell metabolism (23-25). This pathway is also documented to be associated with carcinogenesis in UC (26), and plays a central role in resistance to chemotherapy and radiation therapy in multiple cancer types (27).

In the present study, *PTEN* gene alterations were investigated using fluorescence *in-situ* hybridization (FISH), and the protein expression levels of PTEN, phosphorylation of Ser473 in Akt (pAkt^{Ser473}) and phosphorylation of Thr308 in

Akt (pAkt^{Thr308}) were analyzed using immunohistochemistry (IHC) in UTUC tissues from 65 patients.

Materials and methods

Patients and tissue samples. Patients with confirmed diagnosis of UTUC after ureteroscopic biopsy or computed tomography-guided biopsy, who then underwent nephroureterectomy between January 2007 and October 2014, were included in the present study. Patients with insufficient tissue for complete pathologic review, including the diagnosis of atypical urothelial cells, or without the required clinical data, including demographic and survival information, were excluded. An informed consent form regarding the utilization of residual tissues for medical research was signed at the outpatient visit after explanation by the physician, and all specimens were sent to the tissue bank of Linkou Chang Gung Memorial Hospital (Taoyuan, Taiwan) immediately after the operation at 4°C and then stored at -80°C. The present study was approved by The Human Subject Research Ethics Committee/Institutional Review Board (IRB) of Linkou Chang Gung Memorial Hospital (approval no. 201601555B0). The clinicopathological details of 65 patients with UTUC were analyzed in the study. In total, 30 (46%) patients were male and 35 (54%) were female, with a median age of 77 years (range, 49-98 years). Additionally, 22 slides of normal kidney (19 slides) and ureter (3 slides) tissues were obtained to use as reference. According to guidelines (28,29), nephroureterectomy would be the standard treatment for these patients. As for T3 or T4 lesions, neoadjuvant chemotherapy would be applied if patients were fit enough, followed by surgery if resectable. Adjuvant systemic treatment was initiated if recurrence was found. For all patients, clinical data, including age, sex, history of smoking, alcohol consumption, diabetes mellitus, hypertension, tumor location, histology grade, tumor stage based on the American Joint Committee on Cancer classification (30), metastasis, recurrence and urolithiasis, were recorded (Table I). The overall survival was calculated independently and stratified according to the noteworthy parameters.

FISH for analysis of the *PTEN* gene. Two commercially available dual-color FISH probes (CytoTest Inc.; cat no. CT-PAC101 and CT-LSP042) were designed to detect copy number changes in the region of the human *PTEN* gene, which is located on chromosome 10q23. The probes hybridized to chromosome 10 in both metaphase and interphase and the Locus Specific Probe (LSP), which is around 470 kb in length, exhibited an orange fluorescent signal under the appropriate filters. The other probe, the Chromosome 10 Counting Probe (CCP10), exhibiting a green signal, served as an internal control according to the nature derived from chromosome 10-specific pericentromeric DNA. First, 4- μ m-thick formalin-fixed paraffin-embedded (FFPE) samples were deparaffinized in 3 washes of xylene for 5 min each, and then samples were rehydrated using a descending ethanol series (100, 85 and 70%). Samples were washed in 4X saline sodium citrate (SSC) at room temperature (RT) for 30 min in a rotating shaker, and then were treated with 1 M sodium thiocyanate (NaSCN) at RT overnight. Lastly, slides were washed in distilled water for 5 min. The specimens were digested in 250 μ m 10% pepsin in 0.01 M HCl. Probes

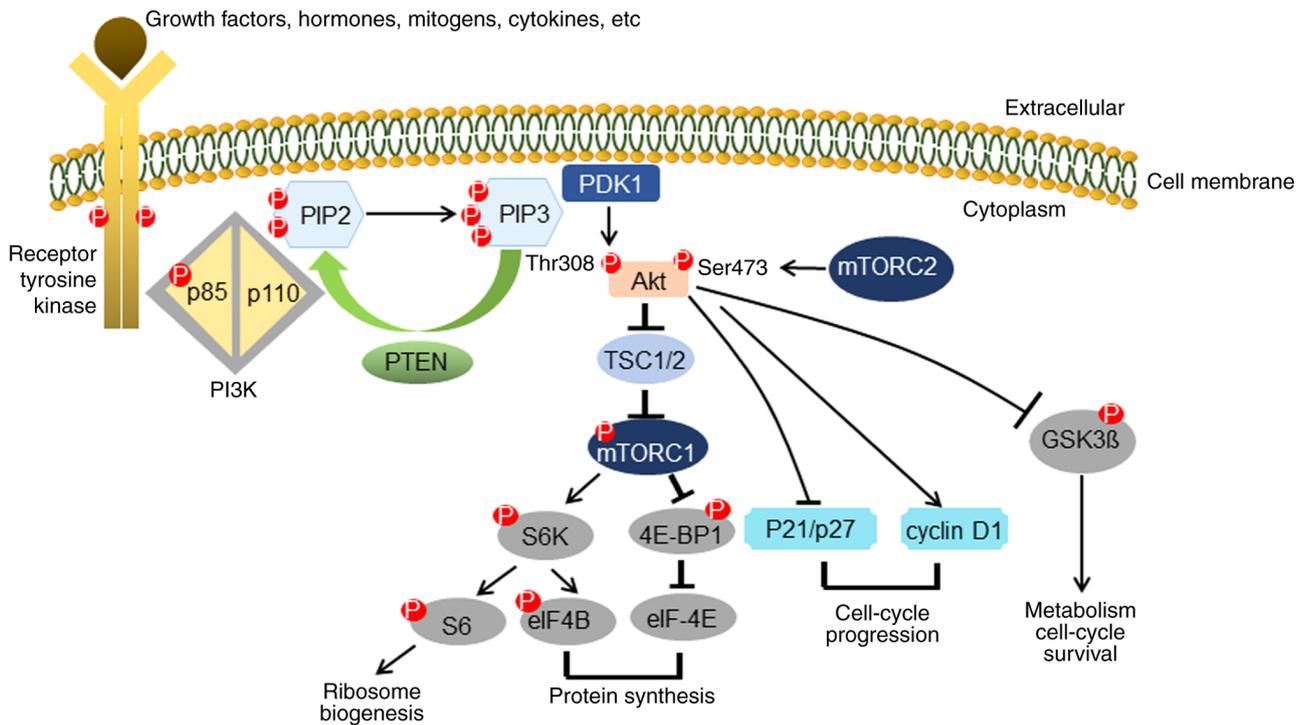


Figure 1. PTEN in the PI3K/Akt signaling pathway. PTEN is a negative regulator in the PI3K/Akt signaling pathway. Akt is recruited to the cell membrane by PIP3, and PDK1 phosphorylates Akt on Thr308. Another cascade is started by mTORC2, which phosphorylates Akt on Ser473 via a positive feedback loop and leads to complete activation of Akt. Via activation of mTORC1, phosphorylation of both the S6K protein and the initial translational factor in eukaryotes, 4E-BP1, will occur. Activated Akt phosphorylates several downstream effectors that regulate a variety of essential processes such as cell growth, cell metabolism, cell survival and protein synthesis. PIP3, phosphatidylinositol (3,4,5)-triphosphate; Thr, threonine; Ser, serine; PDK1, phosphoinositide-dependent kinase-1; mTORC1, mammalian target of rapamycin complex 1; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; S6K, ribosomal protein S6 kinase.

and target DNA were then co-denatured at 82°C for 10 min and hybridized overnight in a 37°C incubator. Post-hybridization washes were performed in 2X SSC at RT for 5 min and in 0.3% NP40/2X SSC at 73°C for 2 min, followed by a 1-min wash at RT in distilled water. All images were captured using a Leica DM2500 fluorescence microscope (Leica Microsystems GmbH) using a magnification of x63 with an ASI CCD camera (CCD-1300DS; Applied Spectral Imaging), and were subsequently analyzed with FISHView EXPO version 5.5 software (Applied Spectral Imaging). To evaluate the *PTEN* copy number, signals in 300 non-overlapping nuclei in each sample were counted using the aforementioned software. DAPI staining of nuclei (using 1 µg/ml at room temperature for 30 min) was performed after resuspension of the cell pellet into absolute ethanol at -20°C (Sigma-Aldrich; Merck KGaA). After staining, the slides were ready for interpretation in reference to the corresponding hematoxylin and eosin (H&E)-stained tissue identified in the areas of carcinoma. H&E staining was performed by the hospital according to routine protocols and a series of 10 slides for the same patient was retrieved after IRB approval. Heterozygous deletion of *PTEN* was defined as a ratio of *PTEN* signal/centromere 10 probe signal <0.5. In addition, homozygous deletion of *PTEN* was defined as a complete absence of *PTEN* probe signal in >60% of tumor nuclei per sample, but there could be 1-2 *PTEN* signals in adjacent cells.

IHC for PTEN, pAkt^{Ser473} and pAkt^{Thr308}. A total of 65 FFPE UTUC tissue samples were collected between January 2007

and October 2014. The areas of carcinoma were identified by three researchers, including one pathologist, in H&E-stained tissues. IHC staining was performed in the present study using primary antibodies against PTEN (clone D4.3 XP; 1:250 dilution; cat. no. 9188), pAkt^{Ser473} (clone 736E11; 1:200 dilution; cat. no. 3787) and pAkt^{Thr308} (clone 244F9; 1:100 dilution; cat. no. 4056) (all Cell Signaling Technology, Inc.). All 4-µm FFPE UTUC tissues were deparaffinized with xylene and rehydrated in 100, 85 and 70% ethanol (Sigma-Aldrich; Merck KGaA). The procedure was performed according to that reported in our previous study with modifications (31). Antigen retrieval was performed by heating the slides at 95°C in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) for 20 min. Endogenous peroxidase activity was quenched by incubation with hydrogen peroxidase (Thermo Fisher Scientific, Inc.) for 10 min at RT, followed by an ultraviolet block (Thermo Fisher Scientific, Inc.) for 5 min at RT to prevent non-specific background staining. Slides were incubated for 16 h at 4°C with the aforementioned rabbit anti-human PTEN, pAKT^{Ser473} and pAKT^{Thr308} antibodies for each group. After 16 h of incubation, the slides were left for 1 h at RT, followed by primary antibody amplifier Quanto (Thermo Fisher Scientific, Inc.) treatment for 10 min at RT. After application of the secondary antibody for 10 min at RT (UltraVision Quanto Detection System; cat. no. TL-060-QHD; ready to use; Thermo Fisher Scientific, Inc.), the slides were stained using the chromogen 3,3'-diaminobenzidine tetrahydrochloride (Dako; Agilent Technologies, Inc.) for 20 sec at RT. Finally, all slides were counterstained with hematoxylin for 20 sec, dehydrated in 95 and 99% ethanol

Table I. Clinicopathological features of 65 patients with upper tract urothelial carcinoma.

Variables	Values
Median age (range), years	77 (49-98)
Sex, n (%)	
Male	30 (46)
Female	35 (54)
Tumor localization, n (%)	
Renal pelvis	60 (92)
Ureter	4 (6)
Renal pelvis and ureter	1 (2)
Histological type, n (%)	
IUC	42 (65)
PUC	21 (32)
IPUC	2 (3)
pT stage, n (%)	
pTa	16 (25)
pT1	12 (18)
pT2	11 (17)
pT3	20 (31)
pT4	6 (9)
Tumor grade, n (%)	
Low	11 (17)
High	54 (83)
Metastasis, n (%)	
Yes	16 (25)
No	49 (75)
Recurrence, n (%)	
Yes	8 (12)
No	57 (88)
History of smoking, n (%)	
Yes	17 (26)
No	48 (74)
History of alcohol, n (%)	
Yes	9 (14)
No	56 (86)
History of urolithiasis ^a , n (%)	
Yes	13 (20)
No	52 (80)
History of diabetes, n (%)	
Yes	17 (26)
No	48 (74)
History of hypertension, n (%)	
Yes	32 (49)
No	33 (51)
Median follow-up (range), months	96 (30-159)

^aUrolithiasis includes kidney, ureter or bladder stones. pT stage, pathological tumor stage; IUC, invasive urothelial carcinoma; PUC, papillary urothelial carcinoma; IPUC, invasive micropapillary variant of urothelial carcinoma.

for 2 min each, and mounted, all at RT. Negative control slides were incubated with PBS at 4°C overnight. Slides from normal kidney or ureter were stained as aforementioned and used as positive controls. All slides were scanned using a high-resolution brightfield APERIO® ScanScope (Leica Microsystems, Inc.) at x40 magnification, and digital images were used for scoring of the immunoreactivity of targeted proteins.

Scoring of PTEN, pAkt^{Ser473} and pAkt^{Thr308} protein expression levels. The IHC score in each case was independently evaluated by one pathologist, one doctor and one researcher. Tumors stained by each marker were evaluated for the location of staining (nuclear or cytoplasmic), extent of staining (percentage of positive cells, 0-100%) and intensity of staining (0, negative; 1, weak; 2, medium; and 3, intense), and a cut-off value was based on the median H-score in tumor (32). Adjacent normal tissues were defined as areas surrounding the tumor in each section with confirmation by pathologist in case of uncertainty. To determine the percentage of positive cells and the staining intensity, the H-score was calculated by the sum of the products of the intensity and extent of expression scores, obtaining a value from 0 to 300. For statistical analysis, the final H-score was divided into two scoring categories. First, cases with H-scores ≥ the median were considered to have high expression; by contrast, cases with H-scores < the median were considered to have low expression (Table II). Second, the results for the nuclear to cytoplasmic expression ratio were stratified into four groups: Nuclear high/cytoplasmic high, nuclear high/cytoplasmic low, nuclear low/cytoplasmic high and nuclear low/cytoplasmic low.

Statistical analysis. The expression levels of protein in adjacent normal tissues and UTUCs were compared using non-parametric statistics. The association between *PTEN* gene alternation, protein expression levels and various clinical characteristics, including that between protein expression in UTUCs and pT stage status (low vs. high), were evaluated using χ^2 test or Fisher's exact test (Table III). Two-way ANOVA with Bonferroni's correction was used to compare the differences between normal and tumor cells in the cytoplasm and nucleus, respectively. Patients who were lost to follow-up were censored on the date of the last visit. All significant parameters from the univariate analyses were included in the multivariate analyses. The probabilities of overall survival were calculated using Kaplan-Meier analysis, and log-rank tests were used to compare overall survival between patient groups. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS software version 20.0 (IBM Corp.) or GraphPad Prism version 7.00 (GraphPad Software, Inc.).

Results

PTEN alterations identified using FISH. In total, 60 (92%) tumors were located in the renal pelvis and 4 (6%) tumors were located in the ureter, with 1 (2%) tumor located in both the renal pelvis and the ureter (Table I). A total of 16 (25%) patients presented with Ta, 12 (18%) patients presented with pT1, 11 (17%) patients presented with pT2, 20 (31%) patients presented with pT3 and 6 (9%) patients presented with pT4. In total, 11 (17%)

Table II. Immunohistochemistry distribution in adjacent normal vs. tumor tissues, and association between cytoplasmic and nuclear expression levels.

A, PTEN expression				
Location	Normal tissues, n (%)	Tumor tissues, n (%)	P-value	χ^2 ^a
Nuclear			0.572	0.319
Low	11 (50)	37 (57)		
High	11 (50)	28 (43)		
Cytoplasmic			0.420	0.651
Low	10 (45)	36 (55)		
High	12 (55)	29 (45)		
B, pAkt ^{Ser473} expression				
Location	Normal tissues, n (%)	Tumor tissues, n (%)	P-value	χ^2 ^a
Nuclear			0.072	5.241
Low	10 (45)	43 (66)		
High	11 (50)	22 (34)		
Absent data	1 (5)	0 (0)		
Cytoplasmic			0.003	11.340
Low	9 (41)	50 (77)		
High	12 (55)	15 (23)		
Absent data	1 (4)	0 (0)		
C, pAkt ^{Thr308} expression				
Location	Normal tissues, n (%)	Tumor tissues, n (%)	P-value	χ^2 ^a
Nuclear			0.113	2.510
Low	10 (45)	42 (65)		
High	12 (55)	23 (35)		
Cytoplasmic			0.147	2.104
Low	10 (45)	41 (63)		
High	12 (55)	24 (37)		

^aProtein expression levels between normal vs. tumor tissues based on median H score were analyzed using χ^2 test. p, phosphorylated; Ser, serine; Thr, threonine. Median value to define high or low in each group: PTEN (cytoplasm) normal 105.55/tumor 46.67; PTEN (nucleus) normal 108.05/tumor 37.78; pAkt^{Ser473} (cytoplasm) normal 66.67/tumor 13.33; pAkt^{Ser473} (nucleus) normal 56.67/tumor 22.22; pAkt^{Thr308} (cytoplasm) normal 27.78/tumor 24.44; pAkt^{Thr308} (nucleus) normal 123.33/tumor 26.67.

patients had low-grade tumors, while the remaining 54 (83%) patients had high-grade tumors. A total of 16 (25%) patients had metastasis, and the median follow-up time was 96 months (range, 30-159 months; Table I). *PTEN* gene deletions were found in 33.8% (22/65) of all UTCUs (Table SI). Representative images of the FISH results are illustrated in Fig. S1. Among the specimens with *PTEN* deletion, 18 (81.8%) samples showed heterozygous deletion and 1 sample had a homozygous deletion. Monosomy of chromosome 10 was detected in 3 samples (data not shown). *PTEN* gene translocation was found in 1 of the heterozygous deletion samples. However, there was no significant association between *PTEN* gene alteration, protein expression (Table SI) or clinicopathological parameters (data not shown).

Protein expression levels identified using IHC. Representative patterns of protein expression levels detected by IHC are depicted in Fig. 2, and the results with further subgroup categorization based on the median H score are summarized in Table II. The distribution of protein expression levels between tumor and normal tissues was not significantly different according to the classification of expression levels above or below the median H score, with the exception of cytoplasmic pAkt^{Ser473} protein expression levels, which were lower in tumor tissues compared with those in normal tissues (P=0.003). As for PTEN expression in the nucleus, 57% of tumors and 50% of normal tissues had low expression levels ($\chi^2=0.319$; P=0.572). Similarly, for PTEN expression in the

Table III. Association between clinicopathological features and target protein expression.

Variables	pT stage			Tumor grade		
	pTa-1, n (%)	pT2-4, n (%)	P-value	Low, n (%)	High, n (%)	P-value
Low cytoplasmic PTEN protein expression	11 (17)	25 (38)	0.023	4 (6)	32 (49)	-
High nuclear pAkt ^{Thr308} protein expression	17 (26)	25 (38)	-	3 (5)	39 (60)	0.026
High cytoplasmic pAkt ^{Thr308} protein expression	18 (28)	23 (35)	-	3 (5)	38 (58)	0.031

pT stage, pathological tumor stage; pAkt, phosphorylated Akt; Thr, threonine.

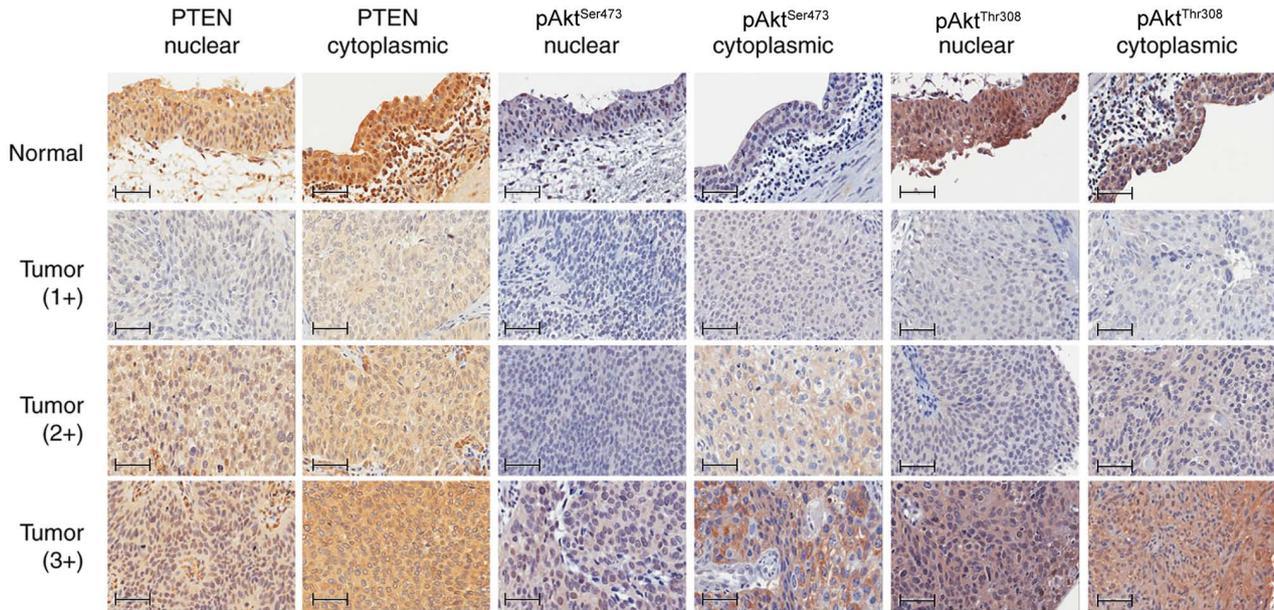


Figure 2. Representative images of nuclear and cytoplasmic protein expression levels in tumor and adjacent normal tissues. The first row shows expression levels in normal tissues. The second, third and fourth row show increasing grades of tumor tissues, including weakly positive (1+), moderately positive (2+) and strongly positive (3+) staining. Magnification, x40. Scale bar, 60 μ m. p, phosphorylated; Ser, serine; Thr, threonine.

cytoplasm, 55% of tumors vs. 45% of normal tissues had low expression levels ($\chi^2=0.651$; $P=0.420$). As for pAkt^{Ser473} expression in the nucleus, 66% of tumors and 45% of normal tissues had low expression levels ($\chi^2=5.241$; $P=0.072$), while a significant difference was found in the cytoplasm with 77% of tumors vs. 41% of normal tissues having low expression ($\chi^2=11.343$; $P=0.003$). As for pAkt^{Thr308} expression in the nucleus, 65% of tumors and 45% of normal tissues had low expression levels ($\chi^2=2.510$; $P=0.113$), and a similar trend was observed in the cytoplasm with 63% of tumors vs. 45% of normal tissues having low expression ($\chi^2=2.104$; $P=0.147$). On the other hand, statistically significant differences were observed in the protein expression levels between tumor and adjacent normal tissues (Fig. 3). PTEN, pAkt^{Ser473} and pAkt^{Thr308} levels were all significantly lower in UTUC tissues compared with those in adjacent normal tissues in terms of H scores. The mean PTEN expression in the cytoplasm between normal and tumor tissues was 104 and 49.53, while that in the nucleus was 109.2 and 43.86, respectively; mean pAkt^{Ser473} expression in the cytoplasm between normal and tumor tissues was 68.2 and 26.9, while that in the nucleus was 77.12 and 31.86, respectively; mean pAkt^{Thr308} expression

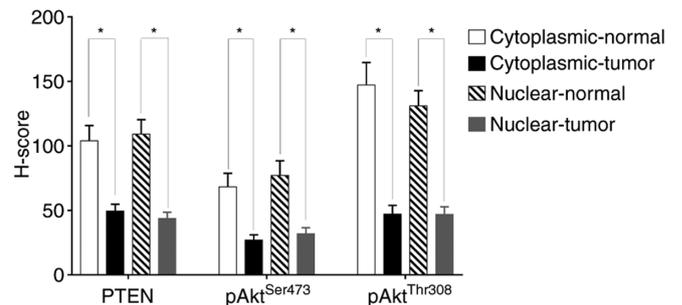


Figure 3. Protein expression levels of PTEN, pAkt^{Ser473} and pAkt^{Thr308} in adjacent normal and upper tract urothelial carcinoma tissues. Protein expression levels in tumor and adjacent normal cells were compared according to locations in the cytoplasm or nucleus. The levels of target protein levels, including PTEN, pAkt^{Ser473} and pAkt^{Thr308}, were all significantly lower in tumor cells compared with those in adjacent normal cells. * $P<0.05$. p, phosphorylated; Ser, serine; Thr, threonine.

in the cytoplasm between normal and tumor tissues was 147.3 and 47.03, while that in the nucleus was 131.1 and 46.95, respectively.

Table IV. Univariate and multivariate analyses for predictors of overall survival.

Variables	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
pT stage		0.01		-
pTa-pT1	1			
pT2-pT4	3.40 (1.34-8.60)			
Metastasis		0.003		0.031
No	1		1	
Yes	3.55 (1.55-8.11)		2.73 (1.10-6.78)	
Cytoplasmic PTEN protein expression		0.016		0.017
High	1		1	
Low	3.14 (1.24-7.95)		3.29 (1.24-8.73)	
Cytoplasmic pAkt ^{Ser473} protein expression		0.019		0.027
Low	1		1	
High	2.71 (1.18-6.21)		2.64 (1.12-6.23)	

pT, pathological tumor; pAkt, phosphorylated Akt; HR, hazard ratio; CI, confidence interval; Ser, serine; Thr, threonine.

Association of PTEN results identified using FISH and IHC. A total of 11/18 samples with heterozygous *PTEN* gene deletion showed lower nuclear and cytoplasmic PTEN protein expression compared with the expression levels of the other samples, and 7 samples had high expression levels. Overall, 2 out of 3 samples with monosomic *PTEN* gene deletion showed lower nuclear and cytoplasmic PTEN protein expression compared with that in the other sample, which had high expression levels. One sample with homozygous *PTEN* gene deletion showed no PTEN protein expression in neither the nucleus or the cytoplasm. There was no significant association between *PTEN* gene alteration and nuclear PTEN protein expression (P=0.434), cytoplasmic PTEN protein expression (P=0.338), nuclear pAkt^{Ser473} protein expression (P=0.423), cytoplasmic pAkt^{Ser473} protein expression (P=0.566), nuclear pAkt^{Thr308} protein expression (P=0.906) or cytoplasmic pAkt^{Thr308} protein expression (P=0.634) (Table SI).

Association of patient characteristics with protein expression levels. Table III summarizes the association of protein expression levels with clinicopathological parameters. Lower cytoplasmic PTEN protein expression levels were significantly associated with advanced T stage (T2-pT4 vs. pTa and pT1, 38 vs. 17%, P=0.023), and expression of nuclear and cytoplasmic pAkt^{Thr308} protein was associated with higher tumor grade (high grade vs. low grade, 60 vs. 5%, P=0.026 and 58 vs. 5%, P=0.031, respectively). However, pAkt^{Ser473} protein expression was not associated with T stage or tumor grade.

Univariate and multivariate analyses of clinical characteristics and protein expression levels. In the univariate analysis, high T stage [P=0.01; hazard ratio (HR)=3.40; 95% CI, 1.34-8.60], metastatic status (P=0.003; HR=3.55; 95% CI, 1.55-8.11), low cytoplasmic PTEN protein expression (P=0.016; HR=3.14, 95% CI, 1.24-7.95) and high cytoplasmic pAkt^{Ser473} protein expression (P=0.019; HR=2.71; 95% CI,

1.18-6.21) were predictive of poor overall survival. However, in the multivariate analysis, only metastatic status (P=0.031, HR=2.73, 95% CI, 1.10-6.78), low cytoplasmic PTEN protein expression (P=0.017; HR=3.29; 95% CI, 1.24-8.73) and high cytoplasmic pAkt^{Ser473} protein expression (P=0.027; HR=2.64; 95% CI, 1.12-6.23) were significantly associated with poor prognosis (Table IV).

Stratification of survival differences according to protein expression subgroups. The results of Kaplan-Meier analysis and log-rank test for overall survival according to clinicopathological parameters and protein expression are presented in Figs. 4 and 5. Survival was poor in patients with high T stage (P=0.006) (Fig. 4A), metastatic disease (P=0.001) (Fig. 4B), low cytoplasmic PTEN protein expression (P=0.011) (Fig. 4C) and high cytoplasmic pAkt^{Ser473} protein expression (P=0.014) (Fig. 4D). The co-expression phenotypes with low cytoplasmic PTEN and high cytoplasmic pAkt^{Ser473} (PTEN^{low}/pAkt^{Ser473/high}) (P<0.001) (Fig. 5A), low nuclear and/or cytoplasmic PTEN and high pAkt^{Thr308} (PTEN^{low}/pAkt^{Thr308/high}) (P=0.024) (Fig. 5B), low nuclear and/or cytoplasmic PTEN and high pAkt^{Ser473} (PTEN^{low}/pAkt^{Ser473/high}) (P=0.016) (Fig. 5C) and low cytoplasmic PTEN with high pAkt^{Ser473} and pAkt^{Thr308} (PTEN^{low}/pAkt^{Ser473/high}/pAkt^{Thr308/high}) (P=0.001) (Fig. 5D) were demonstrated to have unfavorable impacts on overall survival.

Discussion

In the present study, lower protein expression levels of PTEN, pAkt^{Ser473} and pAkt^{Thr308} in either the cytoplasm or nucleus were observed in UTUC compared with those in adjacent normal tissues (P<0.001; Fig. 3). In addition, high T stage, metastatic disease, low cytoplasmic PTEN protein expression levels, high cytoplasmic pAkt^{Ser473} protein expression levels, co-expression of low cytoplasmic/nuclear PTEN and high

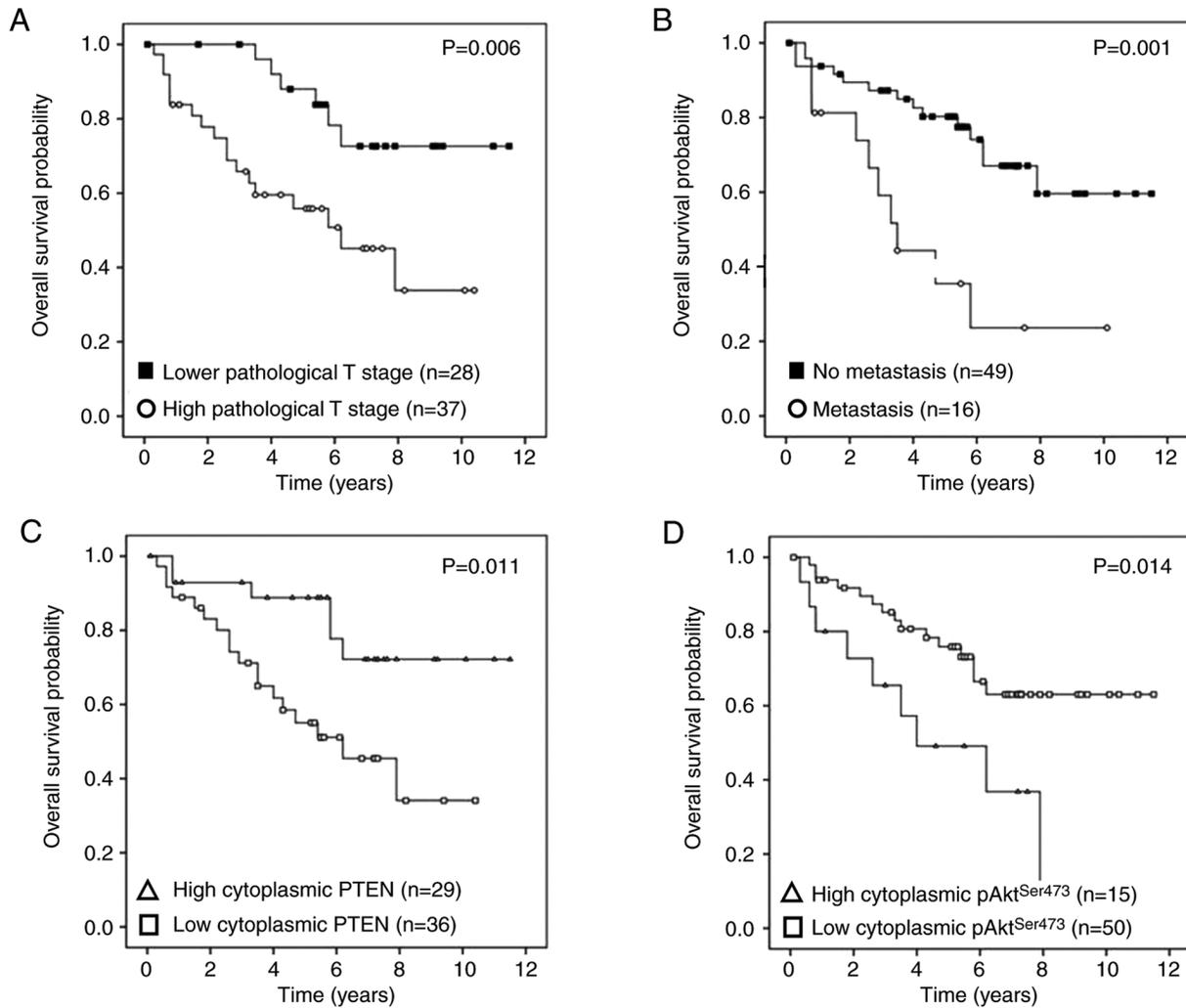


Figure 4. Kaplan-Meier curves and log-rank tests for overall survival according to clinicopathological parameters and protein expression levels. Patients with (A) high pathological T stage, (B) metastasis, (C) tumors that expressed low cytoplasmic PTEN protein expression levels (D) and tumors with high expression levels of cytoplasmic pAkt^{Ser473} protein were significantly associated with decreased overall survival. p, phosphorylated; Ser, serine; Thr, threonine; T, tumor.

cytoplasmic pAkt^{Thr308} and high pAkt^{Ser473} had unfavorable impacts on overall survival (Figs. 4 and 5).

Based on the results from the paired samples analyzed using IHC, the PTEN protein expression levels in either the cytoplasm or nucleus were significantly higher in adjacent normal tissues compared with those in tumor tissues (all $P < 0.001$; Fig. 3). Therefore, alterations in gene structure were further investigated, and the majority of cases presented *PTEN* gene heterozygous deletions, with only one case showing a homozygous deletion, which resulted in the absence of PTEN protein expression.

Nevertheless, a previous study demonstrated that *PTEN* deletion in bladder cancer was significantly associated with recurrence in the Ta stage of disease and with progression in the T1 stage of disease (10). The present study focused on UTUC, and a high proportion of *PTEN* gene deletions (22/65; 33.8%) were observed in UTUC tissues. A previous report by Rieken *et al* (33) revealed that loss of PTEN protein expression was rare, and was associated with an aggressive phenotype, high tumor grade, high tumor stage and metastasis in UTUC. Further association with poor overall mortality was also documented. Although the lower number of cases showing loss of

PTEN protein expression in Western countries contrasts with the higher rate in Taiwan (34), the results regarding patient outcomes were similar in the present study.

In addition, the correlation between the location of proteins in tumor cells and patient survival rate was further analyzed. Notably, it was found that patients with low cytoplasmic PTEN and high pAkt^{Ser473} expression levels (the PTEN^{low}/pAkt^{Ser473}/high group) had significantly shorter overall survival compared with that of other groups ($P < 0.001$). Similarly, patients with low nuclear and/or cytoplasmic PTEN and high pAkt^{Thr308} expression levels (the PTEN^{low}/pAkt^{Thr308}/high group) had significantly shorter overall survival compared with that of other groups ($P = 0.024$). Moreover, patients with low nuclear and/or cytoplasmic PTEN and high pAkt^{Ser473} expression levels (the PTEN^{low}/pAkt^{Ser473}/high group) also showed significantly shorter overall survival compared with that of other groups ($P = 0.016$). Patients with low cytoplasmic PTEN and high pAkt^{Ser473} and pAkt^{Thr308} expression levels (the PTEN^{low}/pAkt^{Ser473}/high/pAkt^{Thr308}/high group) had significantly shorter survival compared with that of other groups ($P = 0.001$) (Fig. 5). When patients lost PTEN expression, regardless of location in the cytoplasm or nucleus, this was significantly

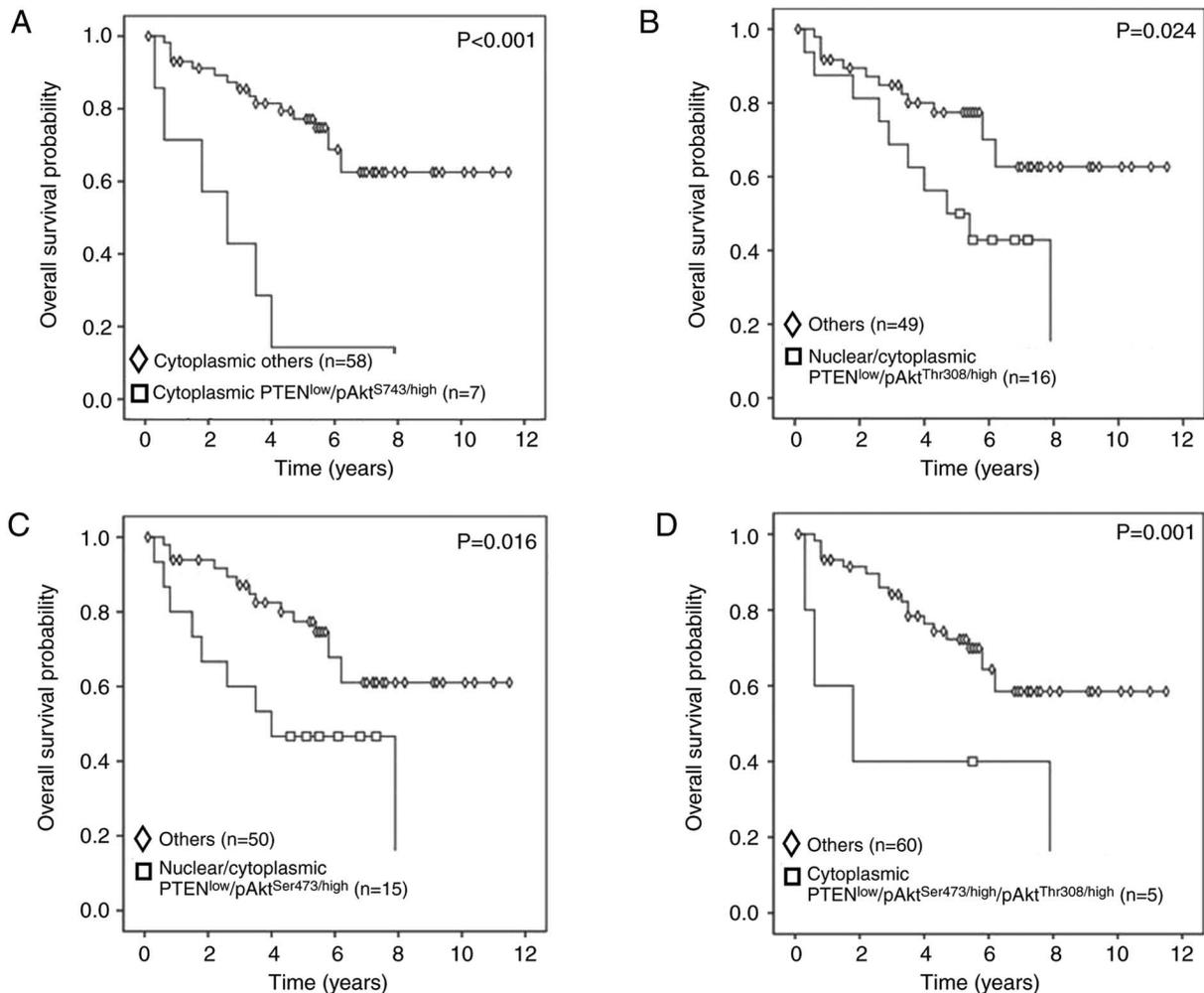


Figure 5. Kaplan-Meier curves and log-rank tests for overall survival according to protein co-expression. (A) Patients in the cytoplasmic PTEN^{low} and pAkt^{Ser473/high} group had significantly shorter survival compared with those in other groups, including cytoplasmic PTEN^{low}/pAkt^{Ser473/low}, cytoplasmic PTEN^{high}/pAkt^{Ser473/low} and cytoplasmic PTEN^{high}/pAkt^{Ser473/high} (P<0.001). (B) Patients in the nuclear and/or cytoplasmic PTEN^{low} and pAkt^{Thr308/high} group had significantly shorter survival compared with those in other groups, including nuclear/cytoplasmic PTEN^{high}/pAkt^{Thr308/high}, nuclear/cytoplasmic PTEN^{low}/pAkt^{Thr308/low} and nuclear/cytoplasmic PTEN^{high}/pAkt^{Thr308/low} (P=0.024). (C) Patients in the nuclear and/or cytoplasmic PTEN^{low} and pAkt^{Ser473/high} group showed significantly shorter survival than those in other groups, including PTEN^{high}/pAkt^{Ser473/high}, cytoplasmic PTEN^{low}/pAkt^{Ser473/low} and cytoplasmic PTEN^{high}/pAkt^{Ser473/low} (P=0.016). (D) Patients in the cytoplasmic PTEN^{low} and combined pAkt^{Ser473/high} and pAkt^{Thr308/high} group had significantly shorter survival than those in other groups, including PTEN^{low}/pAkt^{Ser473/low}/pAkt^{Thr308/high}, PTEN^{low}/pAkt^{Ser473/high}/pAkt^{Thr308/low}, PTEN^{low}/pAkt^{Ser473/low}/pAkt^{Thr308/low}, PTEN^{high}/pAkt^{Ser473/low}/pAkt^{Thr308/high}, PTEN^{high}/pAkt^{Ser473/high}/pAkt^{Thr308/low}, PTEN^{high}/pAkt^{Ser473/low}/pAkt^{Thr308/low} and PTEN^{high}/pAkt^{Ser473/high}/pAkt^{Thr308/high} (P=0.001). p, phosphorylated; Ser, serine; Thr, threonine; T, tumor.

associated with less favorable survival outcomes. Additionally, to the best of our knowledge, homozygous deletion of PTEN is uncommon in urothelial cancer (10), and the present study is the first to report this.

Contrary to expectations, the protein expression levels of pAkt^{Ser473} and pAkt^{Thr308} were lower in tumor samples compared with those in normal tissue samples (Fig. 3). These results coincide with those of a study by Munari *et al* (35), in which 99 archival FFPE tissues were evaluated for the expression status and prognostic significance of members of the mTOR signaling pathway in UTUC. Significantly higher expression levels of PTEN and pAkt were found in benign urothelium tissues compared with those in paired tumor samples. A possible reason for this could be that the prevalence of PTEN loss in tumors varies between 8 and 36% of cases (33), and activation of Akt phosphorylation sites is regulated by different downstream pathways.

PTEN is a tumor suppressor gene, and dephosphorylation of PIP3 into PIP2 can prevent hyperactivation of Akt (11). According to the findings of Makboul *et al* (36), decreased PTEN protein expression levels were significantly associated with high-grade tumors in UC and with poorly differentiated squamous cell carcinomas. A number of studies have indicated that PTEN deletion was more prevalent in the nucleus compared with that in the cytoplasm (37,38). Loss of cytoplasmic PTEN expression was primarily observed in the pT2-pT4 stages of UC, which was documented in 77% (10/13) of UC cases (39). In addition, several studies demonstrated that the active Akt protein is an important regulatory factor in cancer cells, giving rise to uncontrolled proliferation without apoptosis (26,40). For example, the activation of Akt is primarily driven by the phosphorylation of two residues, Thr308 and Ser473, which are located in the activation loop and in the C-terminal hydrophobic motif of the protein, respectively (41). The site

of Ser473 phosphorylation is known to be associated with tumor formation, and its phosphorylation may be triggered by mTORC2 activation (17,42). However, protein activation mediated by phosphorylated Thr308, which in turn is regulated by PDK1, is considered necessary and sufficient to stimulate Akt signaling in cells; therefore, phosphomimetics are commonly used to study the biology of Akt signaling, although they may be insufficient in clarifying the mechanism of the whole Akt signaling pathway. Gallay *et al* indicated that pAkt^{Thr308} could be a diagnostic marker of Akt activity (16). Moreover, Akt phosphorylation at Thr308 is associated with human non-small cell lung cancer (43) (Fig. 1).

In the present study, both cytoplasmic and nuclear PTEN levels in tumors were compared with those in adjacent normal cells ($P < 0.001$; Fig. 3), and lower expression levels of cytoplasmic PTEN were found in muscle invasive disease (high stage, pT2-4) compared with those in non-muscle invasive disease (low stage, pTa-1; $P = 0.023$; Table II). Furthermore, high expression levels of cytoplasmic and nuclear pAkt^{Thr308} were associated with high tumor grade ($P = 0.031$ and $P = 0.026$, respectively). Similar results from stage I lung adenocarcinoma also revealed that a PTEN(-)/pAkt(+)/pmTOR(+) phenotype was associated with poor overall survival (44). In agreement with these data, the present study showed that low cytoplasmic PTEN protein expression levels were observed in high T stage tissues, and high nuclear or cytoplasmic pAkt^{Thr308} protein expression levels were observed in high tumor grade tissues. Furthermore, it was revealed that high T stage, metastasis, low cytoplasmic PTEN protein expression and high cytoplasmic pAkt^{Ser473} protein expression levels were strong predictors of poor survival in univariate analysis (Table IV). In multivariate analysis, metastasis, low cytoplasmic PTEN protein expression levels and high cytoplasmic pAkt^{Ser473} protein expression levels remained strong predictors. Table II showed the association between clinical features and target protein levels by IHC, which showed no specific association between pAkt^{Ser473}, pAkt^{Thr308} and metastasis. However, patients with high cytoplasmic/nuclear pAkt^{Thr308} possessed high tumor grades in the present cohort. Therefore, it was hypothesized that pAkt^{Ser473} and pAkt^{Thr308} are not directly associated with metastasis. It is also noteworthy that high T stage in the present cohort was not a significant predictor in the multivariate analysis. A possible reason for this phenomenon may be that cisplatin-based chemotherapy works well for patients who progress to locally advanced or metastatic disease as standard first-line systemic treatment. Therefore, in the present cohort with the majority of patients at the Ta-T2 (60%) stages, T stage is not a significant predictor for overall survival in the multivariate analysis. Cha *et al*, Margulis *et al* and Ehdai *et al* (45-47) reported that UTUC with lymph node metastasis was associated with worse cancer-specific and overall survival compared with UTUC without lymph node metastasis. In addition, two other studies have shown that nuclear pAkt expression levels and pathological stage were associated with poor prognosis in UTUC (48,49), supporting the present findings and suggesting that the PTEN/PI3K/pAkt signaling pathway is important in the carcinogenesis of UTUC.

The incidence of UTUC accounts for 5-10% of that of all UCs in Western countries, while the incidence is as high as 30% in Taiwan, and UTUC is associated with consumption

of Chinese herbal medicines containing aristolochic acid (AA) (50). In the present study of 65 patients with UTUC, high T stage, metastasis, low cytoplasmic PTEN protein expression levels, high cytoplasmic pAkt^{Ser473} protein expression levels and co-expression of low cytoplasmic PTEN and high pAkt^{Ser473} were strong predictors of poor overall survival. Munari *et al* (35) and Izquierdo *et al* (48) also reported that high T stage, lymph node metastasis and lymphovascular invasion were significant predictors of tumor progression and increased cancer-specific mortality in UTUC. Moreover, Shin *et al* (44) demonstrated that a PTEN(-)/pAkt(+)/pmTOR(+) result from IHC analysis was associated with poor prognosis in stage I non-small cell lung carcinoma. Although these aforementioned factors are highly associated with cancer prognosis when considered together, they are not synergistic and do not have any effect on their own. Koletsas *et al* (39) found that PTEN expression loss was not associated with Akt activation, suggesting that more complicated pathways could be involved through crosstalk or synergistic effects rather than direct activation (38). By contrast, a study evaluating the mTOR pathway members in patients with UTUC showed that none of the existing biomarkers were useful for predicting tumor progression or cancer-specific mortality (35). These findings implied that differences in ethnicity or environmental factors, such as AA, may make the clinical presentation of the disease variable and unpredictable.

There are some limitations in the present study. First, a couple of clinical factors were also associated with oncological outcomes, for example, lymph node status. However, lymph node dissection is not the routine procedure during nephroureterectomy, especially when the pre-operative image study is negative for lymph node disease in patients with UTUC. Although there were 6 patients (9%) with T4 disease, representing the high-risk group for nodal disease, the present study only incorporated distant metastasis as a predictor of long-term oncological outcome instead of addressing the association with lymph node status. Future studies should include lymph node dissection in the prospective setting. Second, FISH and IHC were performed to quantify the expression levels of targeted proteins; despite meticulous quality control and standardization of every procedure, experimental errors may still have occurred in these tests. Alternative analyses should be performed in future work, including western blotting and ELISA for determination of protein expression levels, in order to investigate associations with other notable targets. If further studies showed a significant association with the mechanism of invasion or proliferation, the levels of target proteins before and after systemic treatment could also be explored. The present study showed the preliminary results of the hypothesis, indicating that low PTEN expression with upregulation of phosphorylated Akt^{Ser473} and Akt^{Thr308} may be associated with poor overall survival in UTUC and may be applied as predictors for outcome stratification before surgery. However, in real world practice, it is hard to obtain a large enough sample size of patients with UTUC before surgery for tissue sampling. If technical advancements reach a level of improved accuracy in regard to small tissue volumes, it may be helpful to predict the outcome of surgery or to enable more personalized planning prior to surgery for patients with UTUC with positive markers.

To the best of our knowledge, the present study is the first to evaluate the association of *PTEN* gene alteration and the co-expression of PTEN, pAkt^{Ser473} and pAkt^{Thr308} with clinicopathological parameters and outcomes in UTUC. The present results suggest that patients with negative protein expression patterns should receive further personalized follow-up and adjuvant treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WHW, LCL and STP conceived and designed the study. KJY, LCL, YJP, CKC and YTC analyzed and interpreted the data. WHW, KJY and LCL drafted the initial manuscript, and YJP, STP and CKC critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by The Human Subject Research Ethics Committee/Institutional Review Board (approval no. 201601555B0) of Linkou Chang Gung Memorial Hospital (Taoyuan, Taiwan). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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