

## HER-2/AKT Expression in Upper Urinary Tract Urothelial Carcinoma: Prognostic Implications

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**Abstract.** Aim: To assess HER-2 and p-AKT expression in upper urinary tract urothelial carcinoma (UTUC) in order to determine their value as prognostic factors of tumour progression and cancer-specific survival. Patients and Methods: One hundred consecutive UTUC patients were retrospectively included, between 1990-2004, in 4 tissue microarrays for immunostaining. Median follow-up: 33.03 months. Results: Positive HER-2 expression was found in 10 cases and cytoplasmic p-AKT expression in 84 cases; the expression intensity was strong: 30 cases, moderate: 28 and weak: 26. Nuclear p-AKT expression was found in 6 patients: 1 with strong, and 5 with moderate intensity. Nuclear p-AKT expression was an independent factor for tumour progression (HR=4.145,  $p=0.013$ ), together with grade (HR=4.557,  $p=0.009$ ) and stage (HR=2.085,  $p=0.003$ ). In cancer-specific survival analysis, nuclear p-AKT expression (HR=4.268,  $p=0.017$ ), together with grade (HR=5.214,  $p=0.035$ ) and stage (HR=2.666,  $p=0.002$ ) were identified as independent prognostic factors. Conclusion: Nuclear p-AKT expression together with stage and grade constitute independent prognostic factors for tumour progression and cancer-specific survival.

Upper urinary tract urothelial carcinoma (UTUC) is an infrequently occurring tumour, with an incidence of 0.7-1.1 per 100,000 population per year in Europe, and has increased slightly over the last 30 years (1). It represents 5% of all patients with urothelial cancers (2). Eighty percent of UTUCs are detected after bladder cancer diagnosis. Two

thirds of these patients are at risk of developing other urothelial tumours in the future (1). Radical surgery, such as nephroureterectomy or partial ureterectomy, is the accepted treatment for localized UTUC. Pathological stage and tumour grade are associated with distant metastasis, but they are insufficient to predict the individual outcome of these tumours (3). More accurate knowledge regarding the biological behavior of tumours would allow tailored treatment schedules to be offered to patients, in an attempt to increase survival and reduce morbidity.

The expression or activation of epidermal growth factor receptor ERB-B is altered in many epithelial tumours, and clinical studies indicate that they have important roles in tumour aetiology and progression (4). The ERB-B family comprises 4 receptors: EGFR (HER-1), HER-2 (ERB-B2), HER-3 and HER-4. All members have an extracellular ligand-binding region, a single membrane-spanning region and tyrosine-kinase-containing domain. Ligand binding to ERB-B receptors induces the formation of receptor homo- and hetero-activation of the intrinsic kinase domain, resulting in phosphorylation on specific tyrosine residues within the cytoplasmic tail. These phosphorylations serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signalling pathways (5). The signal transduction pathways activated by pEGFR play important roles in various cellular processes, such as cell proliferation, differentiation, adhesion, migration and apoptosis (6).

HER-2 has been shown to be important in tumour growth and development (7). Activated HER-2 stimulates the phosphoinositide-3 kinase (PI3K)/AKT pathway. The activation of this pathway initialises the recruitment of different adaptor proteins, which bind to different phosphotyrosine residues of the cytoplasmic tail of EGFR, and continues with a high network of enzymes, proteins and small-molecule secondary messengers (8). Activated AKT (p-AKT) can be internalized in the cellular nucleus and can modulate the function of numerous substrates related to cell

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cycle progression. Several mediators within HER-2 and PI3K/AKT have been studied in urological solid tumours (9, 10). Dysregulation of this pathway has been suggested to contribute to the pathogenesis and progression of different human malignancies resulting in enhanced invasiveness, migration, proliferation and prolonged survival of somatic cells (11). In this study, HER-2 and AKT expression in UTUC was assessed in order to determine their value as a prognostic factor of tumour progression and cancer-specific survival.

## Patients and Methods

**Patients.** One hundred consecutive patients with UTUC who underwent nephroureterectomy (n=98) or partial ureterectomy (n=2) at the Hospital Clinic of Barcelona between 1990 and 2004 were retrospectively included in this study. The only exclusion criterion was the lack of tissue from the archive blocks for inclusion in the microarray. The median age of the patients was 69.8 years (range 45-101 years) and the median overall follow-up time was 33.03 months (range 0.3-182.7 months). The distribution per stage and grade was as follows: 14 pTa, 24 pT1, 21 pT2, 28 pT3, 13 pT4 and 9 grade I, 44 grade II and 47 grade III. The location of the tumour was the renal pelvis in 75 patients and the ureter in 25. Thirty patients with UTUC had a previous history of bladder cancer. Twenty-nine of them had non muscle-invasive bladder cancer (19 Ta and 10 T1). There was only one case of muscle-invasive bladder cancer which underwent radical cystectomy before developing UTUC. The pathological analysis of the bladder revealed a pT2 N0 tumour. This patient developed UTUC after 26 months of surveillance, but presented with no tumour progression after 72 months of follow-up. Patients were followed-up postoperatively at 3-month intervals for the first year, and 6-month intervals for the next 2 years, and if they were disease-free after 3 years, follow-up examinations were made yearly. Abdominal and pelvic CT Scan, cytology and cystoscopy were used in the patient follow-up.

Tumours were considered to be progressing when distant metastasis or pathological nodes developed during the follow-up period. Tumours were graded and classified according to the World Health Organisation (12) and the TNM classification of the International Union Against Cancer (13). Samples were obtained under institutional review board-approved protocol.

**Tissue specimens and tissue microarrays (TMA) construction.** Haematoxylin/eosin-stained sections from the formalin-fixed paraffin-embedded cases of UTUC were reviewed and a representative tumour area was selected for each case. Two 2 mm tissue cores were taken from each targeted lesion and placed into four TMA paraffin blocks. First, second and third TMA comprised a cohort of 30 individual tumour samples (60 cores each) and the fourth TMA contained 10 individual tumour samples (20 cores each).

TMA tissue blocks were cut 5 µm thick, and a representative section of each block was stained with haematoxylin and eosin for tumour verification.

**Immunohistochemistry analysis and evaluation.** The following antibodies were used in this study: rabbit polyclonal anti-human c-erbB-2 Oncoprotein (ref. A0485; Dako (Denmark), dilution 1:700)

and rabbit polyclonal phospho-AKT antibody (ref. 9277; Cell Signaling (Boston, USA), dilution 1:50).

Four-micron paraffin-embedded sections were used for immunohistochemical examination. Only cases with 2 preserved cores were considered for evaluation. After paraffin removal and hydration, antigen retrieval for c-erbB-2 was performed using a pressure cooker in 10 mM citrate buffer (pH 6.0). Slides were then incubated with the primary antibody for 30 min at room temperature and rinsed. Peroxidase-labelled polymer conjugated to antirabbit method (DAKO EnVision+ System; Dako Corporation, Boston, MA, USA) was used to detect antigen-antibody reaction. Sections were visualized with 3,3'-diaminobenzidine as a chromogen and counterstained with Mayer's hematoxylin.

Positive and negative controls were included in each slide run. Positive controls were carried out for c-erbB-2 and p-AKT consisting of breast carcinoma, according to the manufacturer's recommendations. Negative controls were carried out by omitting the primary antibody.

Staining evaluation was carried out by two independent observers. The agreement between both observers was >90%. Cases of disagreement were reviewed jointly to reach a consensus score. Immunostaining of HER-2 showed a membranous pattern. For p-AKT, staining was found in the cytoplasm and nucleus.

HER-2 staining was scored according to the Herceptest protocol (14) and considered positive when >10% of tumour cells showed moderate or strong membranous staining. The average score considered was the maximum expression of two scores. The evaluation of p-AKT expression was based on the method described by Pantuck *et al.* (15), which considered staining intensity as 0: negative, 1: weak, 2: moderate, 3: strong and staining frequency as a percentage of positive cells. Intensity and frequency staining were evaluated in both cytoplasm and nucleus. For the statistical analysis, nuclear or cytoplasmic p-AKT expression was defined as being present when more than 10% of the tumour cells were stained.

**Statistical analysis.** The probabilities of progression-free survival and cancer-specific survival were calculated using Kaplan-Meier curves. Statistical differences were identified by the log-rank test. Hazard ratios (HRs) and their confidence interval (CIs) were calculated. Spearman test was used for correlations. In the multivariate analysis, forward stepwise Cox regression was performed. Statistical significance was established at a *p*-value of 0.05, and 95% CIs for the item are presented. SPSS 12.0 software (SPSS, ©SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

## Results

**Immunohistochemistry results.** HER-2-positive expression was present in 10 cases (10%). Cytoplasmic p-AKT expression was found in 84 cases (84%). Of these, 30 presented strong intensity, 28 moderate intensity and 26 weak intensity. Nuclear p-AKT expression was found in 6 patients (6%). One of them presented strong intensity and five moderate intensity (Figure 1). Molecular expression according to tumour stage and grade are shown in Table I.

**Correlations between HER-2 and p-AKT.** HER-2 expression correlated with nuclear p-AKT expression ( $R=0.386$ ,  $p<0.001$ ), tumour grade ( $R=0.355$ ,  $p<0.001$ ) and positive

Table I. Molecular expression according to tumor stage and grade.

	Patients	HER-2		Cytoplasmatic p-AKT		Nuclear p-AKT	
		Positive	Negative	Expression	No expression	Expression	No expression
Stage (TNM)							
pTa	14	1	13	11	3	0	14
pT1	24	1	23	21	3	0	24
pT2	21	1	20	18	3	2	19
pT3	28	5	23	24	4	3	25
pT4	13	2	11	10	3	1	12
Grade							
GI	9	0	9	7	2	0	9
GII	44	0	44	38	6	1	43
GIII	47	10	37	39	8	5	42

Table II. Univariate and multivariate analysis for tumor progression and cancer specific survival in patients with UUTC.

Variables	Tumour progression				Cancer-specific survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR	p-Value	HR	p-Value	HR	p-Value	HR	p-Value
Nuclear p-AKT expression	7.213	0.018*	4.145	0.013*	14.413	0.005*	4.268	0.017*
Histological grade	4.605	0.011*	4.557	0.009*	4.612	0.066	5.214	0.035*
Pathological stage	2.963	0.007*	2.085	0.003*	3.632	0.001*	2.666	0.002*
Positive lymph nodes	1.380	0.183	--	--	1.486	0.131	--	--
Cytoplasmic p-AKT expression	0.647	0.437	--	--	1.159	0.830	--	--
Adjuvant treatment	0.576	0.310	--	--	0.197	0.051	--	--
HER-2 expression	0.424	0.293	--	--	0.360	0.254	--	--

lymph nodes ( $R=0.287$ ,  $p=0.005$ ). Cytoplasmic expression of p-AKT correlated with nuclear p-AKT expression ( $R=0.204$ ,  $p=0.045$ ). p-AKT nuclear expression correlated with tumour grade ( $R=0.245$ ,  $p=0.017$ ).

**Tumour progression.** The two cases treated with partial ureterectomy did not develop tumour progression. Overall, 28 patients developed tumour progression after a median overall follow-up of 33.03 (0.3-182.7) months. The median time of tumour progression was 26.07 (0.6-182.7) months. Thirteen patients had positive lymph nodes at the time of diagnosis. They received adjuvant treatment (platin-based regimen), but all of them developed tumour progression during the follow-up. Five-year disease-free progression of the series was 68.1%.

Results from the univariate analysis show that stage, grade and nuclear p-AKT expression are prognostic factors for tumour progression (Table II). Tumour progression-free survival analysis showed a significant difference between patients with tumours which expressed nuclear p-AKT and those that did not (Figure 2). The 5-year tumour progression-

free survival was 67.6% vs. 0% ( $p<0.001$ ) for negative and positive p-AKT, respectively. For histological grade and pathological stage, the 5-year tumour progression-free survival was 100%, 85.3% and 33.2% for grade I, II, III and 95.2%, 80%, 81%, 36% and 25.2% for pTa, pT1, pT2, pT3 and pT4, respectively (Figure 2).

In the multivariate regression analysis, the independent predictive variables for tumour progression were histological grade (HR 4.557,  $p=0.009$ ), pathological stage (HR=2.085,  $p=0.003$ ) and nuclear p-AKT expression (HR 4.145,  $p=0.013$ ) (Table II).

**Cancer-specific survival.** The two cases treated with partial ureterectomy did not die as a result of cancer. Overall, 20 patients died due to UTUC during the surveillance period. Five-year cancer-specific survival of the series was 72.9%.

Nuclear p-AKT expression and pathological stage were indicative factors of cancer-specific survival in the univariate analysis (Table II). Among the molecular markers, a comparison of the 5-year cancer-specific survival showed that only patients with nuclear expression of p-AKT had

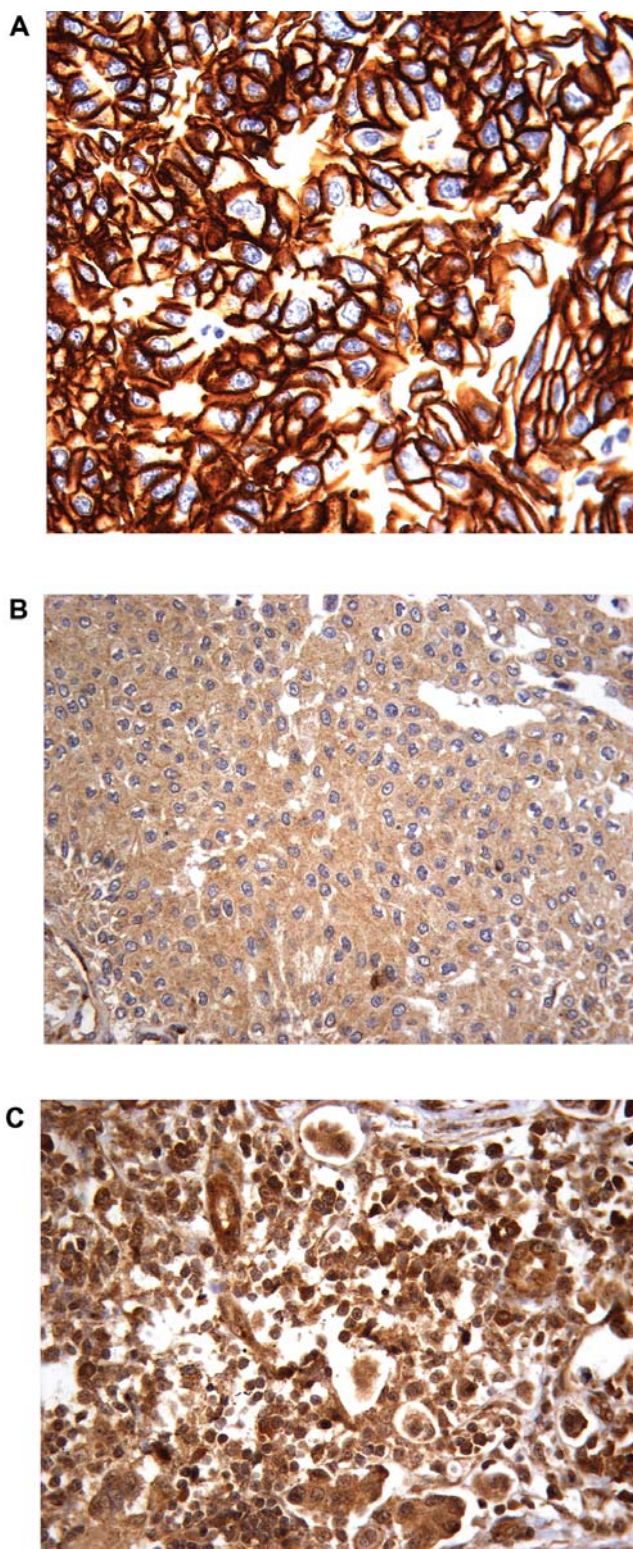


Figure 1. HER-2 and p-AKT expression in UTUC samples. A: Positive HER-2 expression. B: Positive cytoplasmic p-AKT expression (70% of the whole sample was stained with an intensity of 2). C: Positive nuclear p-AKT expression (80% of the whole sample was stained with an intensity of 3). The images also shows cytoplasmic expression of p-AKT).

statistically significantly lower probability of cancer-specific survival (0% vs. 78.3%) ( $p < 0.001$ ). The 5-year cancer-specific survival was 100%, 93% and 44.5% for histological grade I, II, III and 100%, 95.2%, 89.5%, 52% and 18.3% for pTa, pT1, pT2, pT3 and pT4, respectively (Figure 2).

In the multivariate regression analysis, the independent predictive variables of cancer-specific survival were histological grade (HR 5.214,  $p = 0.035$ ), pathological stage (HR 2.666,  $p = 0.002$ ) and nuclear p-AKT expression (HR=4.268,  $p = 0.017$ ) (Table II).

## Discussion and Conclusion

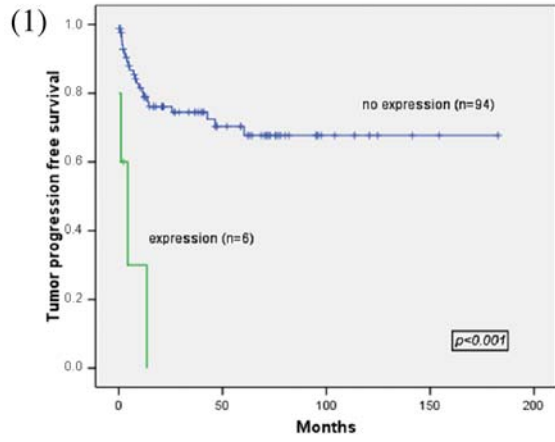
UTUC is considered to have a worse prognosis than urothelial bladder cancer. In the patient series presented here, one quarter of these patients presented with tumour progression after a 4-year follow-up. Since tumour stage and grade are the only established prognostic factors for UTUC, this study focused on a search for biomarkers to improve and personalise prognosis of this disease.

The AKT pathway is de-regulated in many types of cancer (11). AKT is a serine protein kinase involved in several carcinogenesis mechanisms such as cell survival, protein synthesis, apoptosis, genetic instability and cell cycle. This protein is of considerable importance due to the development of kinase inhibitors that are able to reduce tumour growth successfully. The AKT pathway can be activated, amongst others, by HER-2, a receptor related to tumour growth and development. In this study, p-AKT and HER-2 expression were evaluated by immunohistochemical analysis in a series of 100 UTUC patients in order to determine their value as a prognostic factor of tumour progression and cancer-specific survival.

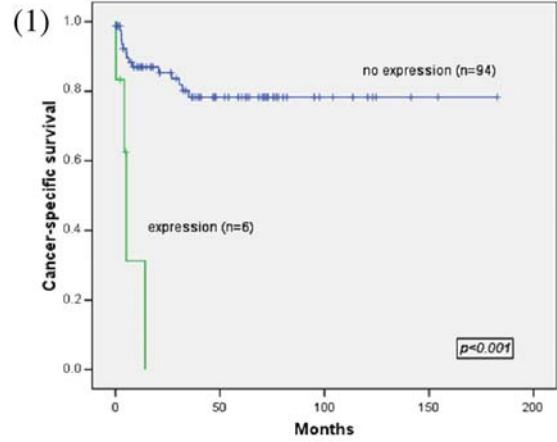
Several studies have explored HER-2 expression and its role as a prognostic factor in bladder cancer, obtaining a discordant percentage of HER-2 staining in their samples and concluding a distinct association between this molecule and its prognostic implication (10, 16-19) (Table III). It is likely that the use of different staining protocols and scoring methods or the small sample size could be responsible for these discrepancies. However, it is notable that several of these studies identified a correlation between HER-2 expression, histological grade (17) and positive lymph nodes (19), in accordance with the results in UTUC presented here. In fact, HER-2 receptor is used as a target for the anti-HER-2 antibody (trastuzumab) treatment in many tumours (20). Also in accordance with the results presented here, neither Wulfing *et al.* (19) nor Jimenez *et al.* (18) found any association between HER-2 expression and disease-free or cancer-specific survival.

In contrast, there are only a few published reports in the literature reporting HER-2 expression in UTUC. Tsai *et al.* (9) studied HER-2 expression by immunohistochemical analysis (n=94 patients) and showed that this molecule was

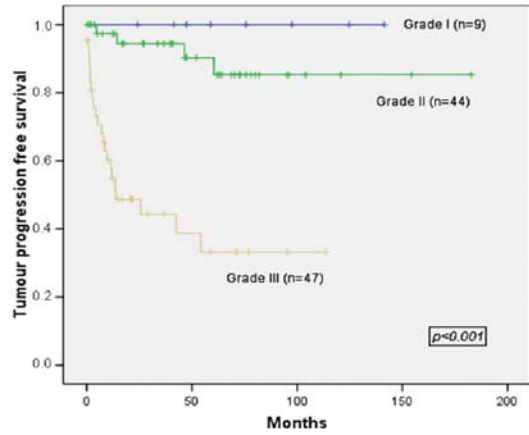
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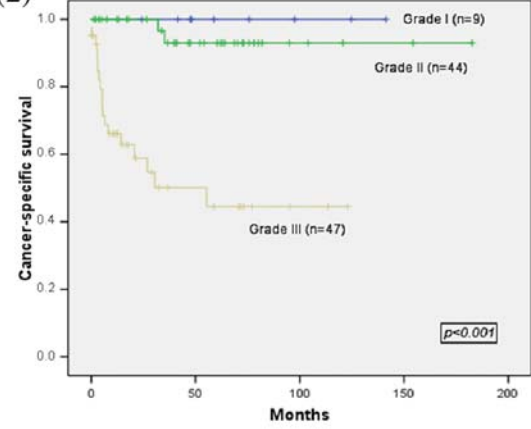
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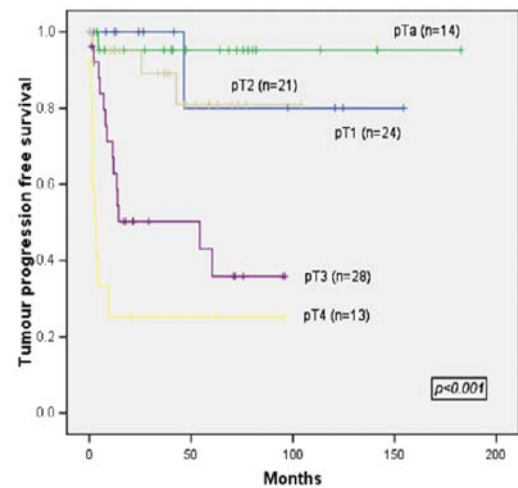
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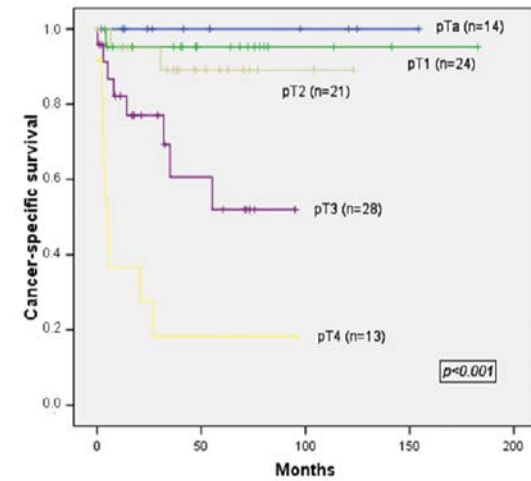


Figure 2. Tumor progression-free survival (A) and cancer-specific survival (B) distribution of patients ( $n = 100$ ) with UTUC according to (1) nuclear p-AKT expression, (2) tumor histological grade and (3) tumor pathological stage.

Table III. *HER-2 expression in several studies of bladder cancer.*

Study	n	HER-2		HER-2 associations		p-Value	Positivity staining criteria
		Expression (% patients)	Tumor HER-2 expression	Related variables			
Jimenez <i>et al.</i> (18)	80	28%	Negative	HER-2 expression in lymph node metastases	$p=0.009$	Complete and strong membranous pattern with moderate intensity Semi-quantitative: > 2/3 cells stained and ++ or +++ staining intensity	
Wester <i>et al.</i> (16)	21	81%	Positive	Distant metastasis	$p=0.002$		
Wulfing <i>et al.</i> (19)	127	74.8%	Positive	Lymph node metastases Distant metastasis	$p=0.06$ $p=0.002$	Herceptest	
Gardmark <i>et al.</i> (10)	90	79%	Positive	Distant metastasis	$p=0.01$	Target score criteria and Herceptest	
Jalali <i>et al.</i> (17)	75	37.3%	Positive	Tumour grade	$p=0.002$	Food and Drug administration approved criteria	

over-expressed in 13.8% of the UTUC samples. These authors showed that HER-2 overexpression was significantly associated with tumour invasiveness. The data presented here are similar, since HER-2 overexpression was found in 10% of the samples and this was correlated not only with tumour stage but also with tumour grade and positive lymph nodes. Moreover, Tsai *et al.* (9) described in univariate and multivariate analyses that tumour stage and HER-2 expression were independent predictors of disease progression, cancer-specific and overall survival. In contrast, this study did not find HER-2 expression to be an independent factor of either tumour progression or cancer-specific survival. It is likely that differences in the surveillance period, or the small size of the patient series, as well as the use of a tissue microarray platform for the immunohistochemical analysis avoiding the bias of the case-by-case studies, could account for these discrepancies. Interestingly, in this study all the patients who expressed HER-2 had GIII tumours. However, these patients did not show statistical differences in cancer-specific survival and tumour progression compared to those GIII patients who did not express HER-2.

It is widely reported that AKT, a serine protein kinase involved in several carcinogenesis mechanisms, is expressed in renal cell carcinoma (15, 21), but its expression has not been described in either UTUC or bladder cancer. A recent publication including 386 patients of renal cell carcinoma (21) concluded that increased nuclear and cytoplasmic p-AKT expression was an independent prognostic factor for patient survival. In this study of UTUC, no association between cytoplasmic p-AKT expression and tumour progression or cancer-specific survival was found. However, nuclear p-AKT expression was found to be an independent prognostic factor for tumour progression and cancer-specific survival. Those 6 patients whose expressed nuclear p-AKT had tumours higher probability of tumour progression

(HR=4.145) and cancer-specific mortality (HR=4.268). Despite the low number of tumours expressing nuclear p-AKT, this subgroup of patients may be at a very high risk of morbidity, making them a target group for the use of the most aggressive therapies. Nuclear p-AKT expression was correlated with HER-2 expression and histological grade. HER-2 expression was positive in 50% of patients whose tumours expressed nuclear p-AKT. The association between HER-2 and cytoplasmic p-AKT and its prognostic impact has been described in gynaecological cancer (7), but it had never been studied in UTUC.

To the Authors' knowledge, the prognostic significance of nuclear p-AKT in UTUC has not been reported to date. Although nuclear p-AKT expression is infrequent in UTUC samples, this study found that it is able to identify a high-risk subgroup of patients and its staining can be used as a prognostic factor for tumour progression and cancer-specific survival. Consequently, this molecule could be a target candidate for new therapeutic strategies in UTUC. However, these results will require validation in a larger and independent series of patients prior to clinical usefulness. In addition, it is necessary to continue searching for new markers in order to improve our knowledge about the nature, development and behavior of these tumours.

In conclusion, these data suggest that nuclear p-AKT expression in UTUC, together with histological grade and pathological stage, constitute an independent prognostic factor of tumour progression and cancer-specific survival.

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