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Expression Status and Prognostic Significance of mTOR Pathway Members in Urothelial Carcinoma of Urinary Bladder Following Cystectomy

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Abstract

Background—Bladder urothelial carcinoma (UrCa) proclaims high rates of mortality and morbidity. Identifying novel molecular prognostic factors and targets of therapy is crucial. mTOR pathway plays a pivotal role in establishing cell shape, migration and proliferation.

Design—TMA were constructed from 132 cystectomies (1994-2002). IHC was performed for Pten, c-Myc, p27, phosAkt, phosS6, and 4E-BP1. Markers were evaluated for pattern, percent and intensity of staining.

Results—Mean length of F/U was 62.6 months (1-182). Disease progression, overall survival (OS) and disease specific survival (DSS) rates were 42%, 60% and 68%, respectively.

Pten showed loss of expression in 35% of UrCa. All markers showed lower expression in invasive UrCa compared to benign urothelium with the exception of 4E-BP1. Pten, p27, phosAkt, phosS6 and 4E-BP1 expression correlated with pathologic stage (pT; $p<0.03$). Pten, 4E-BP1, and phosAkt expression correlated with divergent aggressive histology and invasion.

PhosS6 expression inversely predicted OS ($p=0.01$), DSS ($p=0.001$) and progression ($p=0.05$). c-Myc expression inversely predicted progression ($p=0.01$).

In a multivariate analysis model that included: TNM stage grouping, divergent aggressive histology, concomitant CIS, phosS6 and c-Myc expression; phosS6 was an independent predictor of DSS ($p=0.03$; HR: -.19) while c-Myc was an independent predictor of progression ($p=0.02$; HR:-.38). In a second model substituting organ confined disease and lymph node status for TNM stage grouping, phosS6 and c-Myc remained independent predictors of DSS ($p=0.03$; HR: -.21) and progression ($p=0.03$; HR:-.34), respectively.

Conclusions—We found an overall downregulation of mTOR pathway in UrCa. PhosS6 independently predicted DSS and c-Myc independently predicted progression.

Keywords

mTOR; Pten; Akt; phos S6; 4E-BP1; c-Myc; p27; urothelial carcinoma; bladder

Introduction

Despite recent multi-disciplinary advances in its treatment, urothelial carcinoma of the bladder (UrCa) continues to carry unacceptably high rates of mortality and morbidity with 10-years survival rate of 40-50% [1]. Variable biologic behavior in UrCa appears to be related to differences in oncogenic pathways alterations. While superficial papillary neoplasms are driven by gain-of-function mutations in oncogenes such as H-RAS and FGFR3, flat carcinoma in situ and muscle-invasive tumors usually carry loss-of-function mutations, affecting tumor suppressor genes such as p53 and phosphatase and tensine homologue (PTEN) [2]. Improvements in understanding UrCa oncogenesis have fueled a current search for biological markers which can be targeted for therapy or bare prognostic significance.

Inactivation of PTEN tumor suppressor gene triggers the phosphatidil inositil-3 kinase (PI3K)-protein kinase B (AKT) pathway that leads to Akt phosphorylation and activation (phos Akt). AKT plays a central role in orchestrating interaction between different growth regulating pathways and represents a major feedback control point [3], [4]. Phos Akt promotes cell cycle progression through $p27^{kip1}$ (p27) depletion [5], cell proliferation through c-Myc up-regulation [6], and protein translation through mTOR activation via its main downstream effectors: phosphorylated S6 protein (phos S6) and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1) [7].

The current study evaluates the expression status and reciprocal interplay of six of the above biomarkers (Pten, phos Akt, phos S6, 4E-BP1, p27 and c-Myc) aiming to be the first to evaluate mTOR pathway status as it relates to outcome in a well characterized uniform cohort of UrCa treated by cystectomy.

Materials and Methods

Patient Cohort and Tissue Microarray Construction

We retrieved 144 cystectomy specimens performed at The Johns Hopkins Hospital between 1994 and 2002. All sections were reviewed for confirmation of original diagnoses and staged, according to the 2002 AJCC-TNM classification [8], by two urologic pathologists (RA and GJN). Paraffin blocks were available in 132 cases, including 120 pure UrCa and 12 UrCa with divergent aggressive histology. The latter included 2 tumors with extensive squamous differentiation, 5 with sarcomatoid features (carcinosarcoma), 1 micropapillary urothelial carcinoma and 1 urothelial carcinoma with plasmacytoid features and 3 undifferentiated carcinomas. TMAs were constructed using a Beecher Instrument (Silver Spring-MD) as previously described by Fedor et al [9]. Triplicate tumor samples and paired benign urothelium were spotted from each specimen. Each TMA spot was further categorized as invasive UrCa, high grade non-invasive papillary UrCa or carcinoma in situ (CIS).

Clinico-pathological Data

All pertinent clinico-pathological data were retrieved from electronic medical records. These included patient demographics and preoperative information such as diagnostic procedure, pre-cystectomy treatment and clinical stage. Follow up data on disease progression, post-operative chemotherapy and/or radiotherapy, disease specific survival and overall survival were also obtained (Table 1). Since all patients underwent cystectomy with curative intent, pelvic recurrence as well as recurrent metastatic disease were considered progression events. Three cases that recurred in urinary tract locations other than bladder (eg. renal pelvis) were excluded from the progression and survival analyses. Both tumor pathological stage (pT) and TNM satge grouping were analyzed during statistical analysis.

Immunohistochemistry

Standard immunohistochemistry (IHC) analysis was performed for mTOR pathway members: Pten, phos Akt, phos S6, and 4E-BP1. IHC analysis was also performed for AKT regulated markers c-Myc and p27.

Immunostaining was performed on formalin fixed paraffin embedded tissue sections using Bond max- Leica autostainer (Leica Microsystems, Bannockburn, IL). Sections were deparaffinized, rehydrated and subjected to heat induced antigen retrieval with a buffer solution using a steamer. Sections were then incubated with appropriate primary antibody. Following the application of a secondary polyclonal rabbit antibody (except for c-Myc, for which we used a CSA kit), slides were developed using 3-3′-diaminobenzidine chromogen and counterstained with hematoxylin. Table 2 lists all pertinent markers information including: vendor, clone, dilution, pre-treatment and incubation conditions and detection kit. TMA spots with artifactual folds or lacking tissue target representation were omitted from further analysis. The latter accounted for any variability in number of total evaluable spots/cases among markers. Tumor and benign TMA spots stained with each marker were evaluated for pattern of staining (nuclear vs cytoplasmic), extent (percent of positive cells) and intensity (0 to 3+ score). A final H-score was generated for each marker as the sum of the products of each intensity category X extent of immunoexpression. H-scores were used during statistical analyses for all markers. In addition, Pten analysis was performed using extent scores. During multivariate analysis, a cut-off was used for phos S6 expression based on mean tumor H-score (H-score ≥ 27) and for c-MYC expression based on the 90th percentile tumor H-score (H-score ≥ 15).

Statistic Analysis—Findings were analyzed using the Stata 9.2 (StataCorp; college Station TX) software package. Equality of population medians among groups was tested using Kruskal-Wallis test for non-parametric one-way analysis of variance by ranks. ANOVA analysis of variance was used when comparing categorical variables. Pairwise correlation coefficients were calculated to test relationship among variables. A Cox regression model was used during multivariate analysis.

Results

Patients Cohort

Mean patient age at cystectomy was 68 y; M:F ratio was 4:1 and mean length of follow-up was 62.6 months (1-182). In our cohort, disease progression rate was 42% (25% local recurrence and 17% distant metastases). Overall survival (OS) and disease specific survival (DSS) rates were 60% and 68%, respectively.

Fifty (38%) patients received pre-operative intravesical therapy. Two patients received mitomycin and the remaining 48 were treated with BCG. Six patients (4%) received neoadjuvant systemic chemotherapy and or neoadjuvant radiotherapy.

The TMA sampling was limited to non invasive UrCa in 27 cases. These included 12 pTa/pTis cystectomies and 15 (11%) higher stage lesions where the invasive component was no longer present in the TMA spot.

Biomarkers Expression Status in Relation to Clinicopathological Parameters

Expression levels of all six mTOR pathway related biomarkers are summarized in table 3 and depicted in figure 1.

Pten—Nuclear and or cytoplasmic Pten expression was evaluated in 119 tumors. Pten expression levels were significantly lower in UrCa compared to benign urothelium (H-score: p=0.0001; extent: p=0.0000). Complete lack of Pten expression was seen in 50/119 (42%) UrCa. Additional 9 (7.5%) tumors showed low Pten expression levels (extent <20%). All corresponding benign urothelium showed high Pten expression (extent >50%).

On univariate analysis, significantly lower Pten expression extent were seen in tumor TMA spots with invasion compared to non invasive spots (p=0.0004) and in tumors of divergent aggressive histology compared to pure UrCa (p=0.009). Pten expression showed an inverse correlation with pT and TNM stage grouping (p=0.0001 and p=0.0004, respectively).

phos Akt—Nuclear phos Akt was evaluated in 113 tumors. Phos Akt expression was significantly lower in UrCa compared to benign urothelium (p=0.0000). Fifty (44%) tumors were negative for phos Akt while additional 20 (18%) UrCa showed low phos Akt expression (H-score ≤5).

On univariate analysis, lower levels of phos Akt expression significantly correlated with presence of invasion at TMA spot (p=0.03) and divergent aggressive histology (p=0.04). Phos Akt expression inversely correlated with pT (p= 0.0316). Non-invasive UrCa (pTa/pTis) had significantly higher levels of phos Akt compared to invasive (pT1+) UrCa (p=0.004).

Phos S6—Cytoplasmic phos S6 was evaluated in 114 UrCa. Fourty-two (37%) tumors were negative for phos S6 and additional 10 (9%) tumors showed low phos S6 expression ((H-score ≤5). Phos S6 expression was significantly lower in UrCa compared to benign urothelium (p=0.0000).

On univariate analysis, phos S6 expression did not correlate with presence of invasion at the TMA spot or with divergent aggressive histology (p=NS). However, phos S6 expression inversely correlated with pT stage (p=0.02). Non-muscle invasive UrCa (pT<2) had higher phos S6 levels compared to pT ≥2 (p=0.0030).

4 EBP-1—Cytoplasmic 4E-BP1 was evaluated in 114 UrCa. Thirty-seven (32%) tumors were negative for 4E-BP1. We found significantly higher levels of 4E-BP1 expression in UrCa compared to benign urothelium (p=0.0000).

On univariate analysis, significantly lower 4E-BP1 expression levels were seen in tumor TMA spots with invasion compared to non invasive spots (p=0.005) and in tumors of divergent aggressive histology compared to pure UrCa (p=0.004). 4E-BP1 expression did not correlated with overall pT or TNM stage grouping (p=NS). A trend toward lower expression levels in non-invasive UrCa (pTa/pTis) was found compared to pT ≥1 (p=0.07).

p27—Nuclear p27 was evaluated in 115 tumors. One hundred and three out of 115 (90%) tumors were positive for the marker. p27 expression levels were significantly lower in UrCa compared to benign urothelium (p=0.002).

On univariate analysis, tumors with divergent aggressive histology had significantly lower levels of p27 (p=0.04). p27 showed an inverse correlation with pT and TNM stage grouping (p=0.04 and p=0.003, respectively).

c-Myc—Nuclear c-Myc was evaluated in 114 tumors. 77/114 (67.5%) UrCa were negative for c-Myc and additional 16 (14%) tumors had low levels (H-score ≤ 5) of c-Myc

expression. C-Myc expression was significantly lower in UrCa compared to benign urothelium (p=0.0000).

On univariate analysis, lower c-Myc expression correlated with presence of invasion at TMA spot (p=0.002).

Correlation among tested biomarkers—A weak but statistically significant positive correlation was present between phos Akt and Pten UrCa expression levels (cc=0.19; p=0.03) as well as between phos Akt and p27 (cc=0.25; p=0.008) UrCa expression levels. Pten and p27 UrCa expression also showed positive correlation (c=0.29; p=0.001). UrCa phos S6 expression showed strong positive correlation with UrCa c-Myc expression (c=0.42; p=0.000) and only weak positive correlation with p27 (c=0.20; p=0.03) UrCa expression levels.

Univariate and Multivariate Outcome Analyses

Clinicopathological parameters—On univariate analysis, TNM stage grouping and presence of divergent aggressive histology significantly predicted DSS, OS and disease progression. Concomitant CIS predicted overall survival (p=0.008). pT stage alone was of borderline significance in predicting outcome (Table 4). When pT stage was categorized as organ confined (pT<3) vs non-organ confined (pT≥3), organ confined status significantly predicted all 3 outcome parameters (DSS, OS and disease progression). Presence of lymphovascular invasion (LVI) as well as nodal status were also predictive of outcome (see table 4). The ratio of number of positive nodes to total number of examined nodes (positive node density) was only of borderline significance in predicting disease progression (p=0.07) and DSS (p=0.06). Preoperative therapy, including neoadjuvant chemo or radiation did not correlate with any outcome parameter (p=NS).

To evaluate the prognostic role of the above clinicopathologic parameters before factoring in the input of mTOR pathway markers, two multivariate models were adopted. The first included TNM stage grouping, presence of concomitant CIS and aggressive histology, TNM stage grouping remained an independent predictor of DSS, OS and disease progression (p=0.001; p=0.001; p=0.007 respectively) while concomitant CIS remained a predictor of OS (p=0.04) in this first model. In a second clinicopathologic parameter model that substituted organ confined status and lymph node status for TNM stage grouping, only nodal status remained an independent predictor of disease progression (p=0.03).

Clinicopathological parameters and mTOR related biomarkers expression—Among all six tested biomarkers, phos S6 was a significant predictor of DSS (p=0.001), OS (p=0.01) and disease progression (p=0.05) while c-Myc expression was a significant predictor of disease progression (p=0.01) but not of DSS or OS (p=NS) on univariate analysis (see Table 4).

In the first of two multivariate analysis models that included: TNM stage grouping, presence of divergent aggressive histology, concomitant CIS, phosS6 and c-Myc expression, phosS6 was an independent predictor of DSS (p=0.03; HR: -.19) while c-Myc was an independent predictor of progression (p=0.02; HR:-.38). In the second multivariate analysis model we substituted organ confined disease status and lymph node status for TNM stage grouping. phosS6 remained an independent predictor of DSS (p=0.03; HR: -.21) amd c-Myc remained an independent predictor of progression (p=0.03; HR:-.34).

Discussion

mTOR pathway is a key regulator of protein translation and cell proliferation that has been shown to be up-regulated in several solid malignancies including thyroid carcinoma [10], small cell carcinoma of lung [11], gastrointestinal tumors [12], [13] and clear cell renal cell carcinoma [14]. The main downstream effectors of mTOR pathway (phos S6 and 4E-BP1) have been shown to be independent predictors of prognosis in several types of solid tumors including renal cell [14], ovarian [15], liver [16-19] and mammary carcinomas [20].

While the search for prognostic biomarkers in UrCa initially focused on cell cycle regulators, p53 and retinoblastoma (RB) alterations [21], [22], more recent efforts have also included tyrosine kinase receptors, mTOR and microenvironment interaction pathways [23] as potential prognosticators. Several studies have addressed the prognostic potential of individual mTOR pathway members in bladder cancer [24-26]. Comprehensive simultaneous assessment of key members of PI3K- mTOR pathway and its downstream affected biomarkers, in relation to clinicopathologic parameters and outcome, in a well characterized cystectomy cohort is needed.

PTEN loss leading to activation of AKT/mTOR axis has been reported in other genitourinary tract tumors such as prostate adenocarcinoma[27], [28], renal cell carcinoma[29] and upper urinary tract UrCa[30]. These findings offered a rational for the limited but promising response to mTOR inhibitors therapy in such settings. However, Yoo et al [31], using a mouse model that conditionally deletes PTEN in urogential epithelium, found AKT/mTOR pathway highly activated in prostate tumors, but not in bladder epithelium. Moreover, PTEN knock-out mice were shown to develop urothelial carcinomas in the upper urinary tract, but not in the bladder [30]. In contrast, synchronous deletions of both PTEN and p53 tumor suppressor genes have been shown to lead to the development of invasive bladder cancer in mouse models [32].

Our findings of frequent loss of Pten (42%) and phos Akt (44%) expression in UrCa are in line with prior studies. Pten and phos Akt loss of expression has been previously reported in up to 50% and 70% of UrCa, respectively, while lower Pten expression has been linked to invasive behavior [24-26]. In our cohort, both Pten and phos Akt expression levels inversely correlated with pT stage and aggressive divergent histology and their loss was more likely to be seen in invasive compared to non-invasive tumor component within a given TMA spot. Our findings of lack of correlation of either Pten or phos Akt tumor expression with disease outcome have also been previously illustrated by Harris et al. and others [26], [33]. The generally lower levels of mTOR pathway members in UrCa with divergent histology suggests downregulation of mTOR pathway in less differentiated urothelial tumors.

The positive but weak correlation between Pten and phos Akt expression is intriguing and may suggest a non-conditional association between the loss of inhibitory effect of PTEN tumor suppressor gene and phos Akt expression (Akt activation) in UrCa. Bose et al [34] found no correlation between Pten and phos Akt expression in mammary carcinoma, despite finding both markers to be altered, suggesting that Akt activation is not always directly linked to PTEN loss.

AKT regulates its downstream target mTOR which in turns operates through two distinct complexes: mTORC1 and mTORC2. mTORC2 directly activates AKT in a feedback fashion while mTORC1 pathway regulates cell growth through its main downstream effectors: ribosomal S6 kinase and 4E-BP1. Activation of mTORC1 is thought to stimulate translation through phosphorilation of S6 and inhibition of 4E-BP1. [35]. Our findings of lower phos S6 and higher 4E-BP1 expression levels in UrCa compared to benign urothelium is consistent with the lower activation of AKT found in our UrCa cohort resulting in an

overall downregulation of mTORC1 downstream events. The inverse correlation between phos S6 expression and tumor pT stage in our cohort is in keeping with our finding of a favorable prognostic effect of higher phos S6 levels in UrCa but is in contrast with prior findings of unfavorable prognostic effect of Phos S6 expression in other solid tumors [19].

Our finding of downregulation of phos S6 in more aggressive UrCa tumors could theoretically be related to their hypoxic tolerant phenotype [36] given prior evidence for downregulation of mTOR pathway through HIF1α activation in hypoxic states [37-39] and given the association of HIF1α overexpression with poor outcome in UrCa [40-44]. We are in the process of evaluating HIF1α expression in the current cohort in relation to mTOR pathway expression hoping to test such hypothesis.

In addition to promoting translation, phos S6 is recognized to repress PIK3-AKT pathway through the inhibition of insulin receptor substrates 1 and 2 (IRS1/IRS2) [7]. Accordingly, we found phos S6 expression, but not 4E-BP1 to correlate with other AKT-regulated members: p27 and c-Myc. In fact, the strongest correlation among pathway members was between c-Myc and phos S6. Furthermore, high c-Myc expression was an independent predictor of disease progression in our cohort. These results are also in keeping with a recently suggested MYC-dependent mechanism for phos S6 translation [45].

Lower p27 expression in our UrCa tumors and its inverse correlation with pT stage and aggressive divergent histology is in agreement with prior reports [46-48]. The significantly positive correlation between p27 and Pten expression levels in our UrCa cohort could be interpreted as additional evidence of a potential oncogenic role for downregulation of p27 in PTEN deficient bladders as it has been recently pointed by Yoo et al [31]. Unlike some of the prior studies on p27 in UrCa [46], [48], we did not find loss of p27 expression to be an unfavorable predictor of disease progression or DSS. One possible reason for the difference could be that these studies had a larger proportion of superficial tumors. Interestingly, recent studies have pointed to a favorable effect on outcome for loss of p27 in UrCa especially in combination with other cell cycle markers [21], [49]. Additional large cohort studies are needed to better resolve the prognostic role if any of p27 in UrCa patients.

In summary, our study represent the first attempt at simultaneous assessment of key members of PI3K- mTOR pathway and its downstream affected biomarkers, in relation to clinicopathologic parameters and outcome. We found phos S6 expression to be an independent predictor of DSS (p=0.03) and c-Myc expression to be an independent predictor of disease progression (p=0.02) in addition to TNM pathologic stage grouping in our cohort. Our intriguing novel findings of a statistically significant prognostic role for phos S6 and c-Myc in a multivariate model that included established clincopathologic prognostic parameters are promising and certainly warrant further confirmation in an independent cystectomy cohort, preferably in a prospective setting and in combination with cell cycle markers evaluation. Additional studies to address potential value of mTOR pathway markers expression in predicting therapeutic response to mTOR inhibitors are also warrented.

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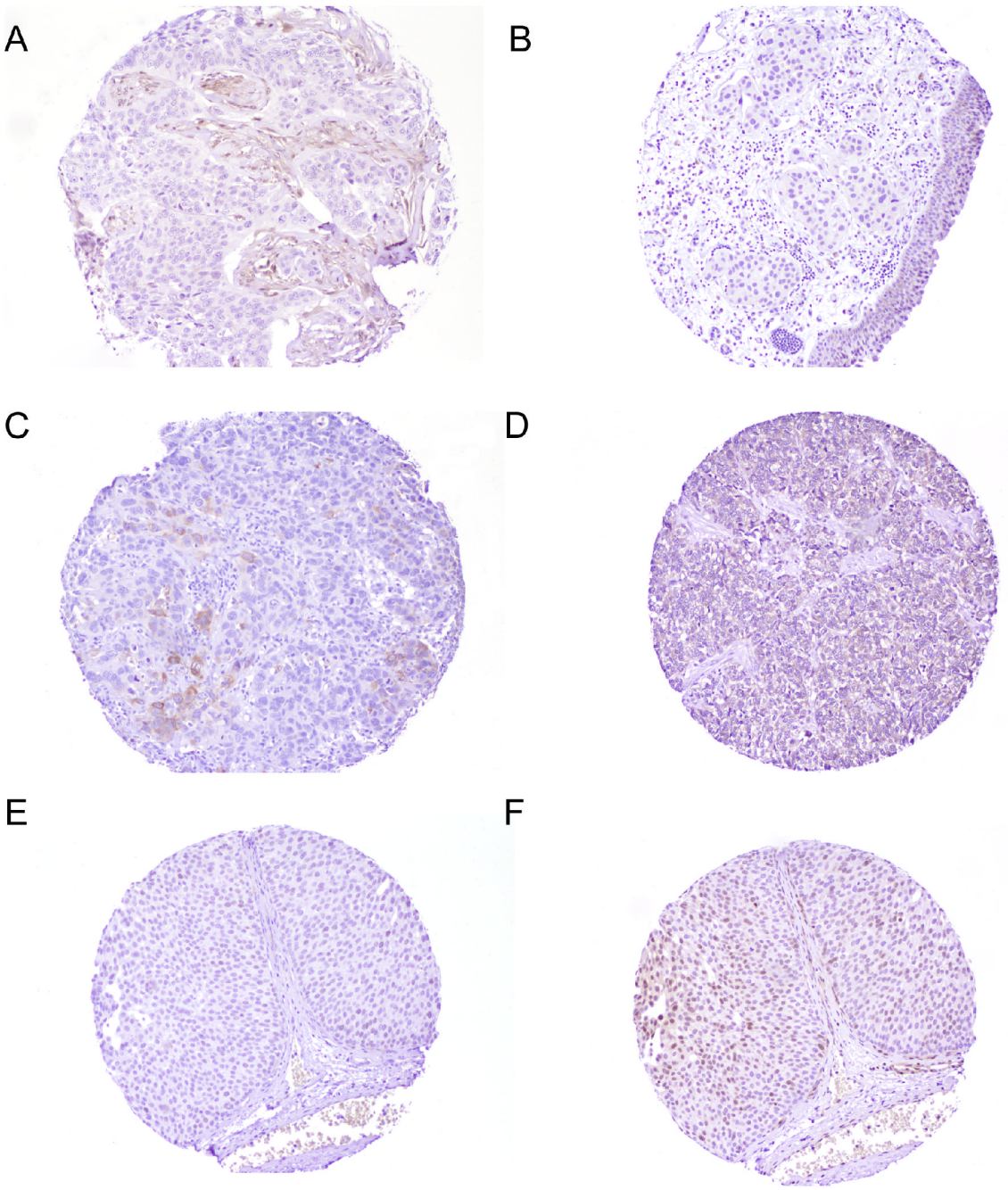

Figure 1.

a: Tissue microarray spot showing lack of Pten immunohistochemical expression in the invasive urothelial carcinoma in contrast to adjacent Pten positive fibroblasts (200× magnification). b: Tissue microarray spot showing loss of phos Akt immunohistochemical expression in the invasive urothelial carcinoma compared to positive staining in the benign overlying urothelium (200× magnification). c: Tissue microarray spot showing focal strong (3+) cytoplasmic phos S6 immunohistochemical expression in invasive urothelial carcinoma (200× magnification). d: Tissue microarray spot showing diffuse moderate (2+) cytoplasmic 4E-BP1 immunohistochemical expression in invasive urothelial carcinoma (200× magnification). e: Tissue microarray spot showing focal weak to moderate (1+ to 2+)

nuclear c-Myc immunohistochemical expression in non-invasive urothelial carcinoma (200× magnification). f: Tissue microarray spot showing multifocal moderate (2+) nuclear p27 and focal cytoplasmic p27 immunohistochemical expression in non-invasive urothelial carcinoma (200× magnification).

Table 1

Demographic and clinicopathological characteristics of 132 cystectomy patients

Characteristic	N (%)
Age (years)at cystectomy	
<60	47 (35.6)
60-70	42 (31.8)
>70	43 (32.6)
Ethnicity	
African American	10 (7)
Caucasian	116 (88)
other	6 (5)
Gender	
Female	26 (20)
Male	106 (80)
Pathologic Stage at cystectomy	
pTa/pTis	12 (9)
pT1	17 (13)
pT2	38 (29)
pT3/pT4	65 (49)
TMA spot sampling	
Non-invasive papillary/carcinoma in situ component	27 (20)
Invasive component	105 (80)
Pre-operative treatment	***56 (42)***
Radiotherapy or systemic chemotherapy	6 (4)
Intravesical therapy	50 (38)

Table 2

Summary of antibodies used in the immunohistochemical analysis of mTOR pathway.

Pten: phosphatase and tensine homologue; p27: $p27^{kip}$; phos Akt: phoporylated Akt; phos S6: phosphorylated S6 protein; 4E-BP1: eukaryotic translation initiation factor 4E-binding protein-1.

	Vendor	Clone	Pre-treatment	Dilution	Incubation	Detection
Pten	Cell Signaling	D4.3	EDTA 45 min	1:50	overnight 4C	PowerVision
phos Akt	Cell Signaling	736E11	EDTA 45 min	1:50	45 min room T	PowerVision
phos S6	Cell Signaling	polyclonal	EDTA 45 min	1:200	overnight 4C	Envision
4E-BP1	ProSci	polyclonal	citrate 25 min	1:250	45 min room T	Envision
c-Myc	Epitomics	Y69	EDTA 45 min	1:300	overnight 4C	CSA kit
p27	Transduction lab	57	citrate 25 min	1:4k	45 min room T	PowerVision

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Table 3

Summary of all six mTOR related biomarkers expression (mean H-score) in the categorized pathological parameters.

pT: pathological stage; Pten: phosphatase and tensine homologue; p27: $p27^{kip}$; phos Akt: phoporylated Akt; phos S6: phosphorylated S6 protein; 4 EBP-1: eukaryotic translation initiation factor 4E-binding protein-1. NS: not significant.

Marker	Benign vs malignant urothelium	Mean H-score	TMA spot histology	Mean H-score	UrCa Histology	Mean H-score	pT	Mean H-score	TNM stage group	Mean H-score
Pten*	Benign	100	Non-invasive	148	Pure UrCa	74	pT0	195	0a/0is	195
							pT1	73	I	61
	Malignant (p=0.0000)	42	Invasive (p=0.0004)	61	Aggressive histology (p=0.009)	34	pT2	79	II	80
							pT3/4	47	III/IV	52
phos Akt	Benign	17	Non-invasive	12	Pure UrCa	7	pT0	23	0a/0is	23
							pT1	7	I	8
	Malignant (p=0.0000)	7	Invasive (p=0.03)	6	Aggressive histology (p=0.04)	3	pT2	6	II	7
							pT3/4	6	III/IV	5
phos S6	Benign	117	Non-invasive	43	Pure UrCa	28	pT0	25	0a/0is	25
							pT1	84	I	75
	Malignant (p=0.0000)	27	Invasive (p=0.1)	25	Aggressive histology (p=NS)	10	pT2	19	II	22
							pT3/4	19	III/IV	21
4E-BP1	Benign	9	Non-invasive	8	Pure UrCa	65	pT0	10	0a/0is	10
							pT1	39	I	42
	Malignant (p=0.0000)	32	Invasive (p=0.0055)	37	Aggressive histology (p=0.004)	29	pT2	25	II	27
							pT3/4	37	III/IV	33
c-Myc	Benign	36	Non-invasive	24	Pure UrCa	2	pT0	5	0a/0is	5
							pT1	14	I	14
	Malignant (p=0.0000)	7	Invasive (p= 0.002)	5	Aggressive histology (p=NS)	8	pT2	7	II	9
							pT3/4	6	III/IV	6
p27	Benign	69	Non-invasive	76	Pure UrCa	53	pT0	63	0a/0is	63
							pT1	93	I	97
	Malignant (p=0.0029)	51	Invasive (p= NS)	47	Aggressive histology (p=0.04)	24	pT2	58	II	65
							pT3/4	36	III/IV	36

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Table 4

Results of univariate analysis of clinicopathological parameters and the expression levels of six mTOR related biomarkers in relations to disease specific survival, overall survival and disease progression. Pten: phosphatase and tensine homologue; p27: $p27^{kip}$; phos Akt: phoporylated Akt; phos S6: phosphorylated S6 protein; 4E-BP1: eukaryotic translation initiation factor 4E-binding protein-1. NS: not significant.

Univariate Analysis			
	Outcome		
	DSS	**OS**	**Progression**
Clinicopathological Parameters			
pT	p=0.080	p=0.083	p=0.061
TNM stage grouping	**p=0.007**	**p=0.024**	**p=0.039**
Organ confined disease status	**p=0.016**	**p=0.021**	**p=0.010**
Lymph node status	**p=0.050**	p=NS	**p=0.010**
Lymphovascular invasion	**p=0.020**	**p=0.002**	p=0.060
Divergent aggressive histology	**p=0.005**	**p=0.029**	**p=0.031**
Concomitant CIS	p=NS	**p=0.008**	p=NS
mTOR Markers			
Pten	p=NS	p=NS	p=NS
Phos Akt	p=NS	p=NS	p=NS
Phos S6	**p=0.001**	**p=0.01**	**p=0.05**
4 EBP-1	p=NS	p=NS	p=NS
c-Myc	p=NS	p=NS	**p=0.01**
P27	p=NS	p=NS	p=NS

Table 5

Two multivariate cox-regression analysis models correlating clinicopathological parameters and expression levels of mTOR related biomarkers with disease specific survival, overall survival and disease progression. Model 1 includes TNM stage grouping while Model 2 includes organ confined disease status and lymph node status as a substitute. Both models include aggressive histology concomitant CIS; Phos S6 and c-myc. DSS: disease specific survival, OS: overall survival; phos Akt: phosphorylated Akt; phos S6: phosphorylated S6 protein. HR: Hazard ratio. 95%CI: 95% confidence interval. NS: not significant.

Multivariate analysis – MODEL 1

	Outcome		
	DSS	**OS**	**Progression**
phos S6	**p=0.03** **HR: -.19** **95%CI: -.37 to -.01**	p=NS	p=NS
c-Myc	p=NS	p=NS	**p=0.02** **HR: -.38** **95%CI: -.70 to -.06**
TNM stage grouping	**p=0.004** **HR: .11** **95%CI: .03 to .18**	p=NS	**p=0.01** **HR: .10** **95%CI: .02 to .18**
Divergent aggressive histology	p=NS	p=NS	p=NS
Concomitant CIS	p=NS	p=NS	p=NS

Multivariate analysis – MODEL 2

	Outcome		
	DSS	OS	Progression
phos S6	**p=0.03** **HR: -.21** **95%CI: -.40 to -.02**	p=NS	p=NS
c-Myc	p=NS	p=NS	**p=0.03** **HR: -.34** **95%CI: -.67 to -.02**
Organ confined disease status	p=NS	p=NS	**p=0.03** **HR: .22** **95%CI: .02 to .42**
Lymph node status	p=NS	p=NS	p=NS
Divergent aggressive histology	p=NS	p=NS	p=NS
Concomitant CIS	p=NS	p=NS	p=NS