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β-Catenin Activation Synergises with PTEN Loss to Cause Bladder Cancer Formation

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Abstract

Although deregulation of the Wnt signalling pathway has been implicated in urothelial cell carcinoma (UCC), the functional significance is unknown. To test its importance, we have targeted expression of an activated form of β-catenin to the urothelium of transgenic mice using Cre-Lox technology (*UroIICRE*+ *β-catenin*$^{exon3/+}$). Expression of this activated form of β-catenin led to the formation of localised hyperproliferative lesions by 3 months, which did not progress to malignancy. These lesions were characterised by a marked increase of the PTEN tumour suppressor protein. This appears to be a direct consequence of activating Wnt signalling in the bladder as conditional deletion of the Apc (Adenomatous Polyposis coli) gene within the adult bladder led rapidly to coincident β-catenin and PTEN expression. This PTEN expression blocked proliferation. Next, we combined PTEN deficiency with β-catenin activation and found this caused papillary UCC. These tumours had increased pAKT signalling and were dependent on mTOR. Importantly in human UCC, there was a significant correlation between high levels of β-catenin and pAKT (and low levels of PTEN). Taken together these data definitively show that deregulated Wnt signalling plays a critical role in driving UCC, and suggests that human UCC which have high levels of Wnt and PI3 kinase signalling may be responsive to mTOR inhibition.

Keywords

β-catenin; PTEN; Urothelial Cell Carcinoma; Bladder Cancer

Introduction

Urothelial cell carcinoma (UCC) of the bladder is the 5th commonest cancer in the world, with 357,000 cases diagnosed yearly on a world-wide basis (Parkin *et al.*, 2005). The majority (75%) of these tumours are non-invasive well differentiated tumours (i.e. pTa, pT1) which can be controlled by transurethral resection of the bladder wall. However, up to 70%

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of the patients with a superficial UCC will have recurrences after its removal, and 10-15% will progress to invasive UCC. Even in those that don't progress, regular surveillance by cystoscopy is required, making bladder cancer one of the most expensive and labour intensive cancers to treat.

A number of genetic and epigenetic alterations involved in bladder tumourigenesis have been identified, including activating mutations in *FGFR3*, and *RAS* family genes, amplification of *ERBB2*, and loss of the *TP53*, *RB1* and *PTEN* tumour suppressors (Cordon-Cardo, 2008; Diaz *et al.*, 2008; Luis *et al.*, 2007; Schulz, 2006). However the role of the Wnt pathway in UCC has yet to be resolved.

The Wnt/ β-catenin signalling pathway plays a crucial role in embryogenesis, cell differentiation and tumourigenesis. Wnts are secreted glycoproteins that act as ligands to stimulate receptor-mediated signal transduction pathways in both vertebrates and invertebrates (Moon *et al.*, 2004). In the absence of a Wnt signal, cytoplasmic β-catenin is phosphorylated and degraded in a complex of proteins. The complex which causes the phosphorylation of β-catenin and thus targets it for degradation is a multi-protein scaffolding complex, consisting of adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), CK1 (casein kinase 1) and axin. Following Wnt pathway activation (through the binding of a Wnt ligand to the frizzled transmembrane receptor), GSK3 is inactivated and β-catenin is no longer phosphorylated and targeted for degradation. As a result β-catenin accumulates in the cytoplasm and enters the nucleus, where it binds to TCF/ LEF family members and transcriptionally regulates Wnt target genes which include cyclin D1 and c-myc (canonical Wnt signalling pathway) (Bienz and Clevers, 2000; He *et al.*, 1998; Polakis, 2000; Tetsu and McCormick, 1999).

Germline and somatic mutations of *APC* are found in the majority of colorectal cancers (Cottrell *et al.*, 1992; Kinzler *et al.*, 1991; Rubinfeld *et al.*, 1993). However, in the case of bladder carcinoma, controversy surrounds the occurrence of somatic mutations in key components of the pathway; Wnt (Bohm *et al.*, 1997; Miyamoto *et al.*, 1996; Stoehr *et al.*, 2002; Urakami *et al.*, 2006a) and β-catenin (Burger *et al.*, 2008; Shiina *et al.*, 2002; Shiina *et al.*, 2001). Many of these studies have demonstrated immunohistochemical upregulation of β-catenin, the key protein in this pathway (Garcia *et al.*, 2000; Kashibuchi *et al.*, 2006; Nakopoulou *et al.*, 2000; Shimazui *et al.*, 1996; Zhu *et al.*, 2000). Urakami and colleagues showed that CpG hypermethylation of Wnt inhibitory factor-1 (Wif-1) was a frequent event in bladder tumourigenesis (Urakami *et al.*, 2006b). Most recently Kastritis and colleagues demonstrated missense (13%) and frameshift (3%) deletions adjacent to the β-catenin binding sites in bladder tumours (Kastritis *et al.*, 2009). They found either *APC* mutations or β-catenin accumulation resulted in shorter disease free interval, and a shorter disease specific survival in multivariate analysis. Similarly, epigenetic silencing of the four secreted frizzled receptor proteins (SFRP), antagonists of the Wnt signalling pathway, has been demonstrated as an independent predictor of invasive bladder cancer (Marsit *et al.*, 2005). In a cohort of 355 patients, a linear relationship between the magnitude of the risk of invasive disease and the number of SFRP genes methylated was observed ($p<0.0004$) with a subsequent reduction in overall survival ($p<0.0003$). Therefore these studies suggest a key role of deregulation of Wnt signalling in bladder cancer, a finding which we test in this current study.

Mutations of the tumour suppressor *PTEN* have been described in many tumours (Salmena *et al.*, 2008), including deletion of the locus in bladder cancers (Aveyard *et al.*, 1999; Teng *et al.*, 1997; Tsuruta *et al.*, 2006). These deletions are absent/rare in superficial tumours, but occur frequently in invasive bladder cancers. Previously, inactivation of PTEN in the murine urothelium has been shown to result in widespread hyperplasia (Tsuruta *et al.*, 2006; Yoo *et*

al., 2006) and recently it has been demonstrated that combined deletion of *Pten* and *Trp53* in the murine urothelium results in aggressive UCC, which is dependent on mTOR signalling (Puzio-Kuter *et al.*, 2009).

In this study, we definitively show that activation of Wnt signalling pathway in the absence of PTEN potently drives UCC *in vivo*.

Results and Discussion

β-catenin overexpression leads to benign hyperproliferation of the urothelium

In order to drive deregulated Wnt signalling, we used mice that carry a dominant allele of the β-catenin gene in which exon3 is flanked by *loxP* sequences (Harada *et al.*, 1999). On addition of Cre recombinase, exon3 is deleted, thus activating Wnt signalling, as this exon contains the residues that are phosphorylated by GSK3β, leading to β-catenin degradation. Thus β-catenin will accumulate and drive Wnt signalling (Moon *et al.*, 2004). To achieve urothelial specific expression of activated β-catenin, these mice were interbred with mice carrying a *uroplakin II (UroII) CRE* transgene (He *et al.*, 2009; Mo *et al.*, 2005). UroII is a protein localised at the apical surface of the urothelium and is important for its permeability barrier function (Zhang *et al.*, 1999). It is expressed throughout the urothelial layers in mice (Mo *et al.*, 2005). The *UroII* promoter has been reported to successfully drive the expression of proteins including SV40 large T antigen and Ha-ras (Mo *et al.*, 2007; Zhang *et al.*, 2001). Previous studies have shown that the $UroIICRE^{+}$ mice exhibit bladder specific recombination (Zhang *et al.*, 1999). To confirm this, and report levels of recombination, we intercrossed the $UroIICRE^{+}$ mice to mice carrying the *Z/EGFP* reporter transgene (Novak *et al.*, 2000). In the *Z/EGFP* reporter mouse, the *Z/EGFP* transgene results in the expression of β-galactosidase by most tissues via a *β-geo* insert, which is flanked by *lox-P* sites. The presence of Cre recombinase results in the excision of the *β-geo*, activating the constitutive expression of GFP. Immunohistochemistry for GFP shows the expression of EGFP in the urothelial lining of the bladder and ureters (Figure 1G) (Novak *et al.*, 2000).

First, we used mice carrying one or two copies of the *β-catenin exon3* allele, to test whether the amplitude of the Wnt deregulation was important, as levels of deregulated Wnt signalling have been shown to be key in other organs such as the mammary gland (Howe and Brown, 2004). To investigate the phenotype of β-catenin activation within the bladder we aged $UroIICRE^{+}$ $\beta\text{-}catenin^{+/+}$, $UroIICRE^{+}$ $\beta\text{-}catenin^{exon3/+}$ and $UroIICRE^{+}$ $\beta\text{-}catenin^{exon3/exon3}$ mice to 3 months of age. In mice carrying one or two copies of the *β-catenin exon3* allele there was a clear phenotype in the bladder epithelium, with all mice developing areas of urothelial hyperplasia (Figure 1A,D). To confirm that this was due to the activation of β-catenin, we performed IHC for β-catenin and saw a marked upregulated of nuclear β-catenin (Figure 1B,E), as well as Wnt target gene c-Myc (data not shown). To confirm the lesions were hyperproliferative we then stained for the proliferation markers Ki67 and BrdU, both of which we found to be upregulated (Figure 1H,I). No differences in proliferation as assessed by BrdU positivity were observed in mice carrying either one or two copies of the β-catenin exon3 allele (Supplementary Figure 1).

Given that mice formed hyperproliferative lesions we predicted that if we aged mice they would develop UCC. To test this we aged $UroIICRE^{+}$ $\beta\text{-}catenin^{+/+}$, $UroIICRE^{+}$ $\beta\text{-}catenin^{exon3/+}$ and $UroIICRE^{+}$ $\beta\text{-}catenin^{exon3/exon3}$ mice to 18 months of age to assess whether they developed cancer. Remarkably, no mice developed UCC within this time course and when bladders from these 18 month old mice were examined they appeared equivalent to those at 3 months with a number of hyperproliferative lesions that had not progressed to cancer (Supplementary Figure 1). There was however a small yet significant increase in the numbers of lesion per bladder ($p<0.001$) (Supplementary Figure 1). This lack

of progression of the lesions highlights that β-catenin activation is not sufficient to drive UCC formation and explains why people and mice that carry germline mutations of *APC* do not develop UCC.

The PTEN tumour suppressor pathway is activated in the bladder lesions

We next investigated the pathways that were constraining tumour progression within the areas of urothelial hyperplasia. UCC's can be broadly separated into two molecular pathways (Luis *et al.*, 2007; Wu, 2005). In one pathway where patients have either *FGFR3* or *HRAS* mutations, they develop superficial papillary disease, which often has a good prognosis. The other tumour subtype has a more aggressive phenotype, leading to muscle invasion and ultimately metastatic disease. These tumours have often lost p53 and/or have an activation of the PI3 kinase signalling pathway. Indeed, a recent study has shown that low PTEN levels correlate with a poor prognosis in human bladder cancer (Puzio-Kuter *et al.*, 2009). This has recently been modelled in the mouse using adenoviral Cre infections into the bladder. Mice either singly mutant for *Pten* or *Trp53* did not develop tumours, however double knockout mice developed metastatic urothelial cancer. Thus we next investigated the expression of candidate molecules from the p53 and PTEN tumour suppressor pathways within lesions from the $UroIICRE^{+}$ $\beta\text{-}catenin^{exon3/exon3}$ mice. Within the lesions we saw very high levels of the PTEN tumour suppressor protein (Figure 1C,F) with minimal pAKT and pMTOR staining (Figure 1J,K) suggesting that the PTEN tumour suppressor was potentially blocking tumour progression. In contrast to a large increase in PTEN, there was only a modest increase in the levels of nuclear p53 (not shown) though one of the targets of p53, p21 was highly upregulated within the lesions of the mice (Figure 1L).

PTEN upregulation acts to block β-catenin driven urothelial proliferation

As the uroplakin cre recombinase is expressed throughout development of the urothelium, it is difficult to assess whether the upregulation of PTEN is a direct consequence of β-catenin accumulation. Therefore we next investigated the consequence of inducibly activating Wnt signalling in the adult urothelium. To do this we used mice carrying the cytochrome p450 inducible $AhCreER^{T}$ transgene. Following administration of both β-napthoflavone and tamoxifen, this yields cre-mediated recombination within the urothelium of the bladder (as well as the intestine). Figure 2A and B demonstrate recombination in the bladder as evidenced by GFP signalling identified by OV100 imaging (ex-vivo) and IHC for GFP in $AhCreER^{T}$ *Z/EG* reporter mice 7 days following induction. To investigate the impact of acutely activating Wnt signalling in the adult bladder we intercrossed $AhCreER^{T}$ mice to mice carrying the inducible knockout Apc^{580S} allele from here on known as Apc^{fl} (Shibata *et al.*, 1997). Remarkably, examination of the bladders from $AhCreER^{T}$ $APC^{fl/fl}$ mice seven days following induction revealed development of urothelial lesions which phenocopied those from our $UroIICRE^{+}$ $\beta\text{-}catenin^{exon3/exon3}$ mice (Figure 2C). Consistent with the activation of Wnt signalling, lesions demonstrated a high level of nuclear β-catenin and again an upregulation in BrdU compared to the surrounding urothelium (Figure 2E,G). Importantly, once again very high levels of PTEN were seen within these lesions (Figure 2I) with minimal upregulation of pAKT (Figure 2K). To confirm this was due to increased levels of Wnt signalling, we also activated Wnt signalling by deleting both copies of GSK3. Bladders from induced $AhCreER^{T}$ $GSK3\alpha^{fl/fl}\beta^{fl/fl}$ mice displayed similar lesions to the $AhCreER^{T}$ $APC^{fl/fl}$ mice, with the accumulation of nuclear β-catenin, BrdU and PTEN (Supplementary Figure 3A-D) (Kemp *et al.*, 2004; MacAulay *et al.*, 2007; Patel *et al.*, 2008). Using this induction regime, mice developed hyperplastic intestinal epithelium, which precluded long term tumour experiments, however in mice aged up to 4 months bladder lesions still remained small and did not progress to cancer, suggesting PTEN was once again blocking tumourigenesis.

To test the role PTEN was having immediately following Apc loss in the urothelium we generated *AhCreER^{T} APC $^{fl/fl}$ PTEN $^{fl/fl}$* mice. 7 days following induction mice again developed urothelial lesions but these were much larger than in *AhCreER^{T} APC $^{fl/fl}$ PTEN$^{+/+}$* mice (Figure 2D). These lesions showed an accumulation of nuclear β-catenin, high BrdU expression and consistent with PTEN deletion, a complete absence of PTEN staining in the lesions (Figure 2F,H,J), and showed strong pAKT(Ser473) upregulation (Figure 2L) To investigate whether the reason for the enlarged lesions was due to hyperproliferation, we examined the number of BrdU positive cells per lesion in both *AhCreER^{T} APC$^{fl/fl}$ and AhCreER^{T} APC$^{fl/fl}$ PTEN$^{fl/fl}$* mice and demonstrated a statistically significantly increase in proliferation when PTEN is lost ($p<0.05$, Mann Whitney Test) (Supplementary Figure 3E). Thus PTEN accumulation following β-catenin activation is acting to limit proliferation. We again were unable to further analyse the *AhCreER^{T} APC$^{fl/fl}$ PTEN$^{fl/fl}$* mice as these mice became ill rapidly after induction due to intestinal disease at day 8

PTEN loss cooperates with β-catenin activation to drive UCC formation

To test whether this block of proliferation by PTEN was suppressing tumourigenesis we intercrossed *UroIICRE^{+} β -catenin$^{exon3/exon3}$* mice, to mice carrying a conditional inactivatable *Pten* allele (where exons 4 and 5 are flanked by *lox p* sites) (Lesche *et al.*, 2002). A recent study using adenoviral Cre delivery to the bladder has shown that deletion of *Pten* alone in the murine urothelium is not sufficient to promote bladder cancer formation (Puzio-Kuter *et al.*, 2009). We confirm this result here, as neither *UroIICRE^{+} Pten$^{fl/+}$* nor *UroIICRE^{+} Pten$^{fl/fl}$* mice developed cancer when aged until 18 months (n=20). Indeed, no phenotypic changes were observed in urothelium between *UroIICRE^{+} Pten$^{fl/fl}$* and wildtype mice (Supplementary Figure 4A-B). To confirm that *Pten* was deleted in these mice we stained for PTEN levels by IHC and found a downregulation of the PTEN protein in bladders from *UroIICRE^{+} Pten$^{fl/fl}$* (Supplementary Figure 4C-D) with similar levels of pAKT(Ser473) staining (Supplementary Figure 3E-F). These data are consistent with our studies within the intestinal epithelium where *Pten* deletion was not sufficient to drive tumourigenesis and only modestly affected the levels of pAKT (Marsh *et al.*, 2008).

To test the cooperation of β-catenin and PTEN loss we generated the following cohorts: UroIICRE^{+} β-catenin$^{exon3/+}$ Pten$^{fl/+}$, UroIICRE^{+} β-catenin$^{exon3/exon3}$ Pten$^{fl/+}$, UroIICRE^{+} *β-catenin$^{exon3/+}$ Pten$^{fl/fl}$*, and *UroIICRE^{+} β-catenin$^{exon3/exon3}$ Pten$^{fl/fl}$* (n=20,16, 24, 21 respectively). We then performed two sets of experiments; first, we harvested mice at 3 months of age and analysed the bladder phenotypes, and second, we aged mice until tumour development. At 3 months of age hyperplastic lesions (scored from 3 H&E cross sections of each mouse, with 3 mice in each cohort) were observed at an increased frequency in doubly mutant mice compared with mice carrying only β-catenin mutation (Figure 3A).

Second, in contrast to the *UroIICRE^{+} β-catenin$^{exon3/+}$ Pten$^{fl/+}$* and *UroIICRE^{+} β-catenin$^{exon3/exon3}$ Pten$^{fl/+}$* mice, the *UroIICRE^{+} β-catenin$^{exon3/exon3}$ Pten$^{fl/fl}$* mice and *UroIICRE^{+} β-catenin$^{exon3/+}$ Pten$^{fl/fl}$* mice rapidly developed symptoms of bladder tumourigenesis; abdominal swelling, haematuria (blood in the urine) and hunching (Figure 3B). On necropsy, we observed bladder tumours (Figure 3C), and histologically, lesions had now progressed to papillary carcinoma (Figure 4A,B). We found no evidence of metastasis in any of our models. These double mutant mice demonstrated no increase in invasiveness. Consistent with *Pten* deletion, the tumours that developed showed complete loss of PTEN protein and now displayed strong activation of pAKT(Ser473) (Figure 4C,D). Tumours also showed a nuclear upregulation of β-catenin and Ki-67 (Figure 4E,F). The number of proliferating cells within tumours (identified by Ki-67 and BrdU IHC) were much higher than levels found in the *UroIICRE^{+} β-catenin$^{exon3/exon3}$* mice (data not shown), possibly explaining why lesions do not progress in the single mutant.

These data are consistent with tumour formation in the bladder being synergistically promoted by Wnt and PI3 kinase signalling. This scenario fits with our previous studies in the intestinal epithelium where Wnt activation or *Pten* loss alone, were not sufficient to induce high levels of pAKT and presumably PI3 kinase signalling (Marsh *et al.*, 2008). However in our bladder model, combination of deregulated Wnt signalling and PTEN loss caused a dramatic increase in pAKT that presumably drives tumour formation. Of the targets downstream of pAKT^{ser473}, we also see a dramatic increase in p-mTOR^{2448} (Figure 4G), suggesting that mTORC activation is a key component of tumourigenesis in this model. Importantly, total levels of AKT and mTOR are unchanged suggesting that loss of PTEN stimulates strong PI3 kinase signalling only once b-catenin is additionally activated within the bladder epithelium. These studies predict that in the absence of PTEN alone, there is sufficiently low endogenous PI3Kinase signalling within the bladder to preclude AKT phosphorylation even in the absence of PTEN. In contrast, a prediction of our data is that activation of the Wnt signalling pathway stimulates PI3 kinase activity; however the increase in PTEN protein blocks most downstream activation of p-AKT and p-mTOR. To examine this a little more closely, we examined a number of candidate ligands and receptors in UroCre B-catenin$^{exon3/exon3}$ mutant that should stimulate PI3 kinase activity and found that Type I insulin growth factor receptor β subunit (IGF-IR β) was upregulated (Figure 4I). This data would therefore suggest that the modest increase in p-AKT/p-mTOR signalling may be contributing to the increase in proliferation within the small lesion. To test this we treated 3 month old *UroIICRE^{+} β -catenin$^{exon3/exon3}$* mice (which at this time have fully established hyperproliferative lesions) with Rapamycin (10mg/kg IP daily) or vehicle for 4 weeks. We were able to demonstrate a consistent downregulation of BrdU positivity/lesion ($p<0.05$) in the Rapamycin treated cohort compared to the vehicle controls (Supplementary Figure 2). Interestingly in tumours arising in double mutant *UroIICRE^{+} β-catenin$^{exon3/exon3}$ PTEN$^{fl/fl}$* bladders, there was a even more marked accumulation of IGF-IRβ (figure 4J), suggesting further stimulation of PI3 kinase signalling.

A recent chemoprevention study by Puzio-Kuter has shown that mTOR inhibition using rapamycin suppresses tumourigenesis in *Trp53/Pten* double knockout tumours (Puzio-Kuter *et al.*, 2009). Consistent with our tumours being dependent on PI3-kinase signalling, there was no upregulation of pERK1/2 in the tumours (Figure 4H). By 12 months of age, a small subset of the *UroIICRE+ β-catenin$^{exon3/exon3}$ Pten$^{fl/+}$* mice and *UroIICRE+ β-catenin$^{exon3/+}$ Pten$^{fl/+}$* mice had developed tumours. This was presumably due to the loss of the remaining *Pten* allele, as staining for PTEN was absent from the tumours (Figure 4K). There has been much debate over the crosstalk between the Wnt and PI3 kinase pathways and it is often proposed that the inhibitory phosphorylation of GSK3 by AKT/PKB may allow the activation of Wnt signalling. If this was the case one would argue that PTEN loss alone should be sufficient to activate Wnt signalling and numerous studies have shown that this is not the case (including our present study) (Salmena *et al.*, 2008). Moreover two key studies suggest that the phosphorylation of GSK3 by AKT does not affect Wnt signalling. First 'knock-in' mice where the AKT phosphorylation sites on GSK3alpha (Ser21) and GSK3beta (Ser9) were converted to alanine did not elevate Wnt signalling (McManus *et al.*, 2005). Second a recent study has shown that the Wnt pool of GSK3 is physically distinct from the AKT pool (Ng *et al.*, 2009). Our data here shows it is only in the complete genetic absence of both GSK3α and GSK3βthat Wnt signalling is deregulated (Supplementary Figure 3).

UroIICRE^{+} β-catenin$^{exon3/exon3}$ PTEN$^{fl/fl}$ UCCs are mTOR dependent

Given mTOR is one of the key tumour promoting pathways downstream of PTEN loss and even in pten wild type lesions which had a modest activation of mTOR there was an impact of rapamycin, we next investigated whether tumours from *UroIICRE^{+} β-catenin$^{exon3/exon3}$*

Pten$^{fl/fl}$ mice would remain dependent on mTOR even if they were fully established. Therefore we treated *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *Pten*$^{fl/fl}$ mice at 6 months of age with Rapamycin (10mg/kg Ip daily) or vehicle (n=3) when mice had a detectable tumour using Visualsonic's Vevo 770 ultrasound. Remarkably we were able to demonstrate regression of tumour bulk between initiation and the end of treatment (Figure 5A-D). All mice on treatment survived the 4 week experiment, however in the vehicle treated mice cohort, 2 mice had to be sacrificed early (7 and 11 days) because of tumour burden. IHC for p-mTOR^{2448} revealed significant upreregulation of this pathway in *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *PTEN*$^{fl/fl}$ mice. However when treated with 4 weeks of rapamycin we noticed regression of the lesions and downregulation of the protein staining, as well as downregulation of 2 of its targets p-4EBP1 and p-S6 Kinase(Thr421/Ser424) (Figure 5E,F, Supplementary Figure 6). We noticed a statistically significant reduction in the BrdU positive cells in the tumours from the rapamycin treated mice compared to the vehicle controls ($p<0.05$, Mann Whitney Test) (Supplementary Figure 7).

Human UCC demonstrate correlation between Wnt activation and PTEN loss

In human urothelial cancer, a number of studies have suggested Wnt signalling is important. Of particular note is the demonstration that nuclear β-catenin is associated with a poor prognosis, and methylation of the inhibitors of Wnt signalling, the SRFPs, act as markers of a bad prognosis (Marsit *et al.*, 2005; Urakami *et al.*, 2006a; Urakami *et al.*, 2006b). Indeed the methylation of these proteins has been suggested as a marker of invasive bladder carcinoma.

We next looked at human bladder UCC using a tissue microarray of 80 cases, 60 UCC (transitional cell carcinomas [TCC]) and 20 benign controls (Folio biosciences, OH, USA). Using the histoscore technique we were able to demonstrate correlation between upregulation β-catenin and loss of corresponding PTEN signal (n=36/56, cc=0.314, $p<0.01$, SPSS v15) as well as upregulation of β-Catenin and pAKT^{Ser473} (n=30/56, cc=0.471, $p<0.001$) (Figure 6A-F) (Kirkegaard *et al.*, 2006). Reassuringly we observed a strong correlation between pAKT^{Ser473} and p-mTOR^{2448} (cc=0.667, $p<0.0001$). This is further indication that loss of PTEN/upregulation of pAKT is essential for Wnt driven UCC to progress.

Our data here suggest that activation of Wnt signalling will strongly cooperate with other mutations that occur in bladder cancer such as PTEN/activation of pAKT to drive carcinoma formation. This may indicate that the significance of Wnt signalling in human bladder cancer has been underestimated, due to the relatively rare nature of *APC* and *β-catenin* mutations. Traditionally up to a quarter of bladder tumours are thought to exhibit nuclear β-catenin (Kastritis *et al.*, 2009). Instead the Wnt signalling pathway may be involved by epigenetic inactivation or mutations in other components of the pathway (e.g. secreted frizzled related proteins). This might suggest that these tumours may be responsive to antibodies that inhibit Wnt signalling through blocking ligand binding such as Frizzled8CRD-hFc (DeAlmeida *et al.*, 2007). Indeed our studies would suggest in those patients where tumours had high levels of β-catenin and p-AKT, the combination of mTOR and Wnt inhibition may be particularly efficious.

Taken together, we are first to examine the causative role of β-catenin in the formation of UCC *in vivo* and provide definitive evidence that activating mutations in the Wnt pathway promote UCC when combined with other tumour suppressor mutations.

Materials and Methods

Mice

Uroplakin II Cre mouse (UroIICRE^{+}) (Zhang *et al.*, 1999) were intercrossed with mice harbouring *β-catenin* $^{exon3/+}$ (Harada *et al.*, 1999) and *Pten* $^{loxP/loxP}$ (Lesche *et al.*, 2002) in combinations as described below. The AhCreER^{T} mice (Kemp *et al.*, 2004) were also utilised and intercrossed with mice harbouring *Z/EGFP* (Novak *et al.*, 2000), *APC*$^{fl/fl}$ (Shibata *et al.*, 1997) and *PTEN*$^{fl/fl}$ (Lesche *et al.*, 2002) mice. AhCreER^{T} (Kemp *et al.*, 2004) mice were intercrossed with *GSK3αβ*$^{fl/fl}$ (MacAulay *et al.*, 2007; Patel *et al.*, 2008) mice. Mice were genotyped by PCR as previously described (Harada *et al.*, 1999; Ireland *et al.*, 2004; Kemp *et al.*, 2004; Lesche *et al.*, 2002; Novak *et al.*, 2000; Shibata *et al.*, 1997; Zhang *et al.*, 1999). Mice were of a mixed background and littermates were used as control mice. All experiments were carried out in accordance with UK animal regulations.

When bladders were excised they were all emptied of urine, before being placed in formalin for overnight fixation before paraffin embedding. All bladders were processed and cut in the same manner by a single histology technician to all standardization.

Rapamycin Treatment

Rapamycin (LC laboratories, Woburn, MA) was provided once daily via i.p. at 10mg/kg *in vivo* for 4 weeks. The i.p. solution was made up as previously described (Namba *et al.*, 2006)

Immunohistochemistry

IHC was performed on formalin fixed, paraffin embedded samples. For each genotype we stained at least 3 samples from different mice and took representative images for this manuscript. We used antibodies against*: Ki-67* (VP-RM04, VectorLabs, 1:100, Citrate buffer and water bath antigen retrieval – 50 minutes at 99oC), *PTEN* (#9559, Cell Signalling, 1:100, Citrate buffer and water bath antigen retrieval – 50 minutes at 99oC), *pAKT(Ser473)* (#3787, Cell Signalling, 1:50, Citrate buffer and microwave antigen retrieval), total *AKT*(pan) (#4685, Cell Signalling, 1:50, Citrate buffer and microwave antigen retrieval), *β-Catenin* (C19220, Transduction Labs, 1:50, Tris/EDTA water bath antigen retrieval – 50 minutes at 99oC), *p21* (M19, Santa Cruz, 1:500, Citrate buffer and water bath antigen retrieval – 50 minutes at 99oC), *p53* (VP-P956, Vector Labs, 1:200, Citrate buffer and microwave antigen retrieval), *GFP* (ab290, AbCam, 1:250, Citrate buffer and microwave antigen retrieval), *mTOR(Ser2448)* (#2976, Cell Signalling, 1;100, citrate buffer and microwave antigen retrieval), total *mTOR* (#2983, Cell Signalling, 1:50, citrate buffer and microwave antigen retrieval), *IGF-IR β* (#3027, Cell Signalling, 1:600, citrate buffer and microwave antigen retrieval), *p-4EBP1* (#2855, Cell Signalling, 1:500, citrate buffer and microwave antigen retrieval), *p-S6 Kinase(Thr421/Ser424)* (#9204, Cell Signalling, 1:100, citrate buffer and microwave antigen retrieval).

Microscopy

Light microscopy was carried out using the Olympus BX51. All images were taken at 20x magnification. For GFP *in vivo* imaging we used the Olympus OV100 system. We imaged mice post-mortem with both skin intact and removed.

Ultrasound

This was performed on live mice using Visualsonic's Vevo 770 (Visulasonics Inc, Toronto, Canada).

Human Tissue Microarray (TMA)

This was purchased from Folio biosciences (OH, USA) and consists of 60 cancer and 20 benign bladder cancer cases with data that consisted of patient sex, age and tumour grade. Slides were scanned using the Aperio slide scanner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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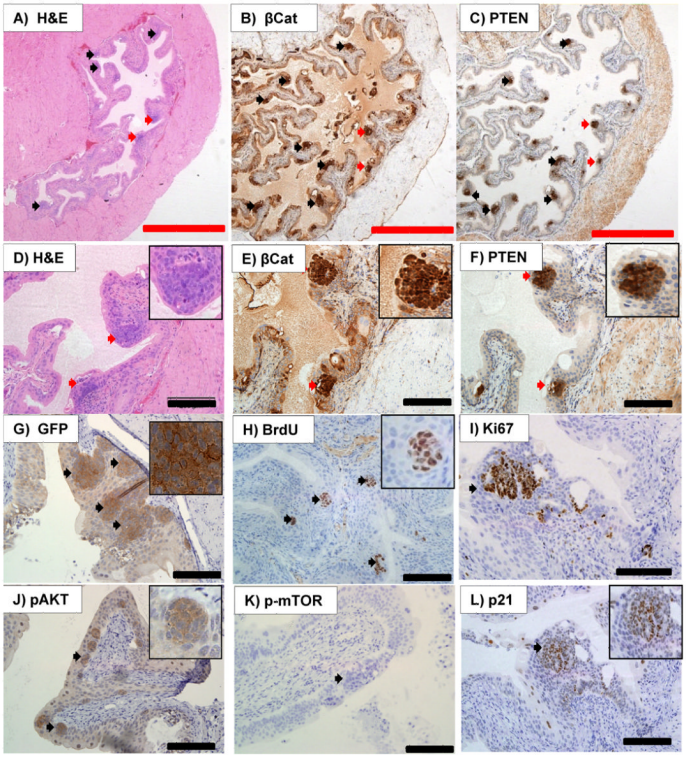

Figure 1. Histology of *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ mice

Histology from 3 month old *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ mice. H&E reveals development of hyperplastic lesions in the urothelium (black/red arrows) (A), of which a close up (red arrows) is seen in (D). Immunohistochemistry shows strong upregulation of nuclear β-catenin (B,E) and PTEN (C,F) in these hyperplastic areas. IHC for GFP reveals evidence of recombination (G). We can see that these lesions show upregulation of markers of proliferation; BrdU (H) and Ki-67 (I). Within these lesions we see minimal pAkt(Ser473) (J) and pmTOR (K) signal. We also notice significant upregulation p21 (L).
Red bar measures 1000 μm (4x magnification), black bar measures 200μm (20x magnification).

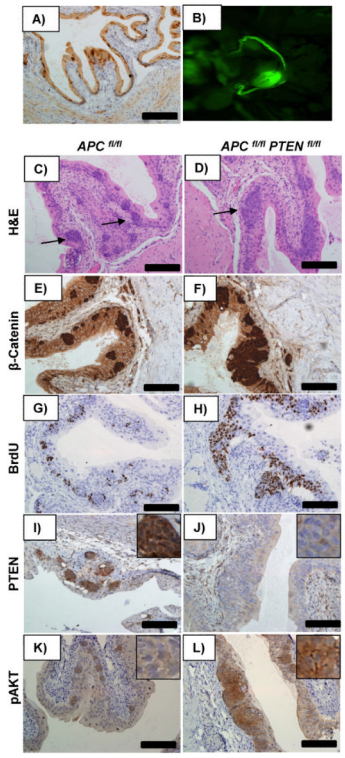

Figure 2. Histology of *AhCreER^T APC $^{fl/fl}$* and *AhCreER^T APC $^{fl/fl}$ PTEN $^{fl/fl}$*

Bladders from *AhCreER^T Z/EG* mice reveal recombination in the bladder as evidenced by GFP signalling, identified by IHC for GFP (A) and OV100 imaging (ex-vivo) (B) and. Bladders from *AhCreER^T APC $^{fl/fl}$* mice reveal similar lesions to our *UroIICRE$^+$ β-catenin$^{exon3/exon3}$* (C), which demonstrate proliferation (G) as well as upregulation of nuclear β-catenin and PTEN staining with minimal pAKT expression (E,I,K). However bladders from *AhCreER^T APC $^{fl/fl}$ PTEN $^{fl/fl}$* mice reveal larger lesions (D) which show further proliferation (H), nuclear β-catenin (F), but this time the absence of PTEN staining in the lesions (J). These lesions demonstrate significant upregulation of pAKT (L). Black bar measures 200μm (20x magnification).

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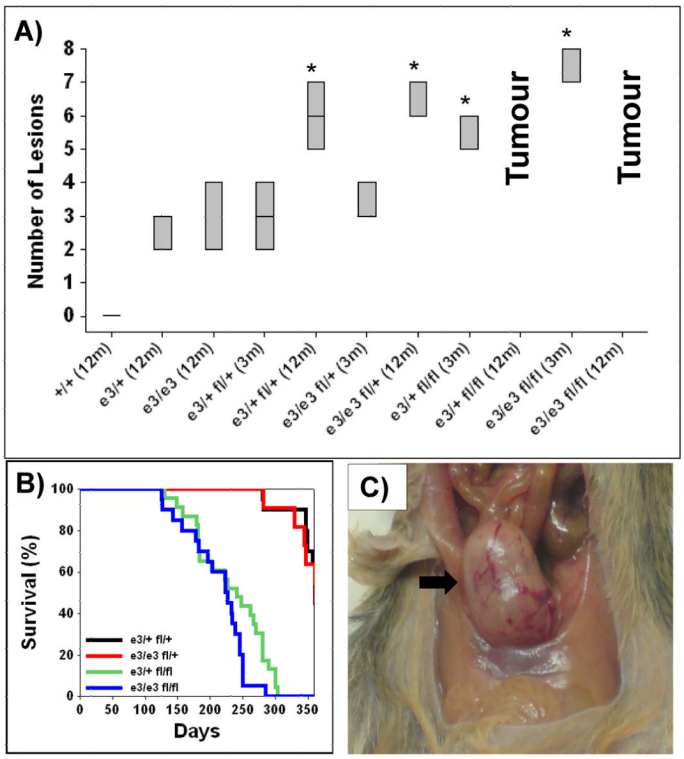

Figure 3. Tumour burden and Survival of *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *Pten*$^{fl/fl}$ mice

Box plot of number of lesions found in urothelium of each cohort at 3 and 12 months (n=3) (A). Cohorts denoted by * demonstrate significantly elevated levels of lesions in comparison to 12 month old *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ ($p<0.05$, Mann Whitney Test). Kaplan Meier curves of tumour free survival of respective double mutant cohorts (B). Photograph of *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *Pten*$^{fl/fl}$ bladder tumour (C).

Abbreviations: e3/e3 (*UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$), e3/+ fl/+ (*UroIICRE*$^{+}$ *β-catenin*$^{exon3/+}$ *Pten*$^{fl/+}$), e3/e3 fl/+ (*UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *Pten*$^{fl/+}$), e3/+ fl/fl (*UroIICRE*$^{+}$ *β-catenin*$^{exon3/+}$ *Pten*$^{fl/fl}$) and e3/e3 fl/fl (*UroIICRE*$^{+}$ *β-catenin*$^{exon3/+}$ *Pten*$^{fl/fl}$)

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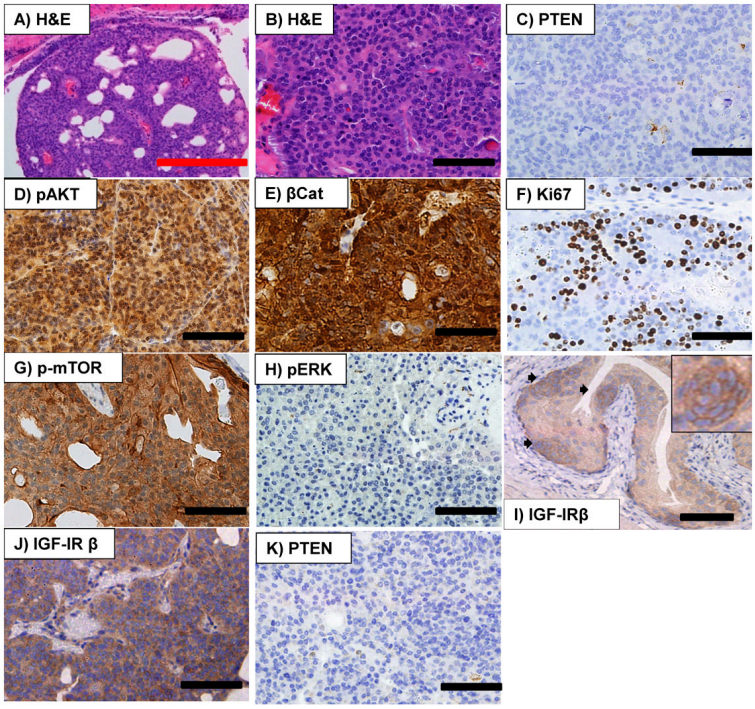

Figure 4. Histology of a *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *PTEN*$^{fl/fl}$ mice

Histology of a *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *PTEN*$^{fl/fl}$ mice reveals urothelial bladder tumour (A,B) with loss of PTEN (C) and upregulation of pAKT(Ser473) in these tumours (D). There is also upregulation of nuclear β-catenin (E), Ki67 (F) and mTOR(Ser2448) (G). We notice no upregulation in pERK1/2 in these tumours (H). Interestingly we see upregulation of IGF-IR β in the *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ lesions as well as the *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *PTEN*$^{fl/fl}$ tumours (I,J). By 12 months of age, a small subset of the *UroIICRE*+ *β-catenin*$^{exon3/exon3}$ *Pten*$^{fl/+}$ mice and *UroIICRE*+ *β-catenin*$^{exon3/+}$ *Pten*$^{fl/+}$ mice had developed tumours, presumably due to the loss of the remaining *Pten* allele (K).

Red bar measures 1000 μm (4x objective), black bar measures 100μm (40x objective).

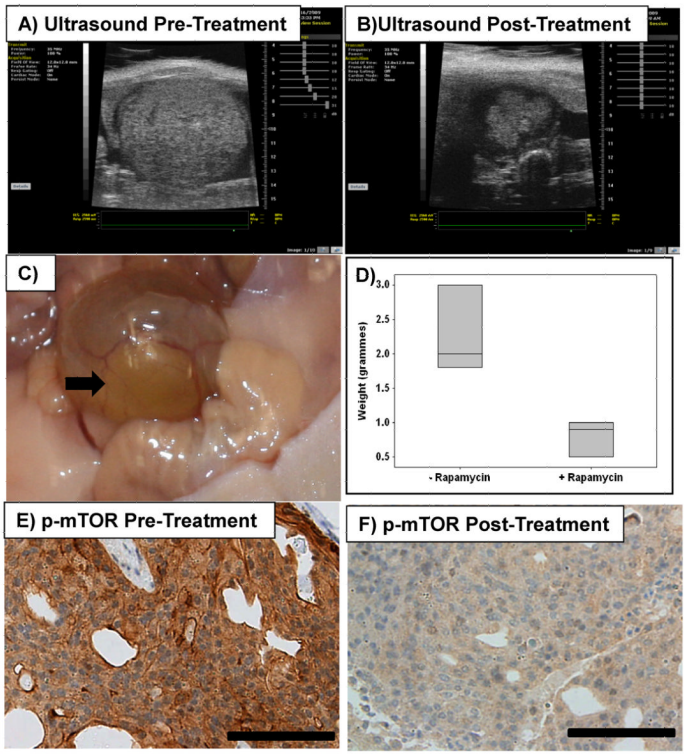

Figure 5. ***UroIICRE*** $^{+}$ ***β-catenin*** $^{exon3/exon3}$ ***PTEN*** $^{fl/fl}$ **mice treated with Rapamycin**

Analysis of *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *PTEN*$^{fl/fl}$ mice treated with 4 weeks of daily IP injections of Rapamycin 10mg/kg. Ultrasound imaging reveals shrinking of the tumour in the treated mice bladders between initiation (A) and end of treatment (B) regimes. We see regression of tumour formation (C) from non treated controls. Boxplot shows that bladders of treated mice have less tumour bulk $p<0.05$, Mann Whitney Test) (D). IHC for pmTOR reveals significant upregulation of this pathway in *UroIICRE*$^{+}$ *β-catenin*$^{exon3/exon3}$ *PTEN*$^{fl/fl}$ mice (E). However when treated with 4 weeks of rapamycin we notice regression of the lesions and downregulation of the protein staining (F).

Black bar represents 100μm (all magnifications at 40x).

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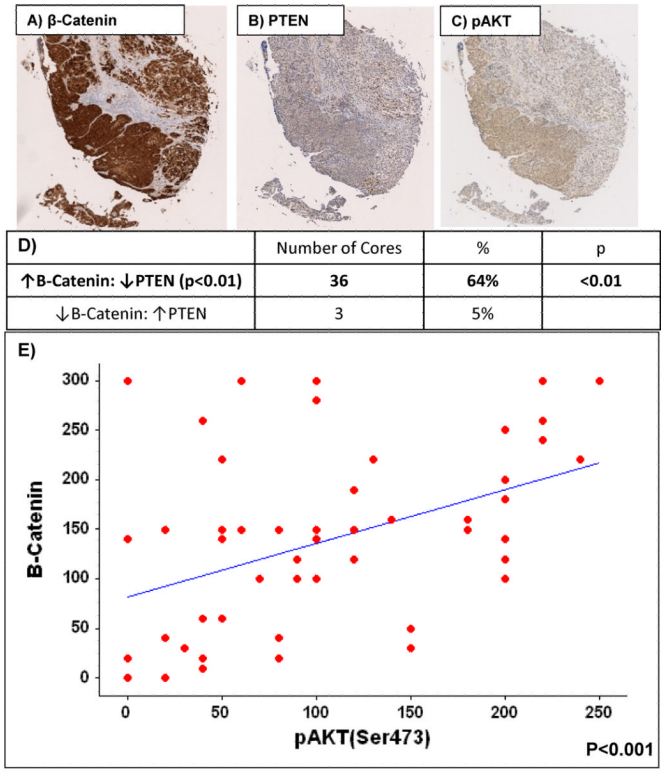

Figure 6. Human Bladder UCC TMA

IHC of human bladder transitional cell carcinoma revealing upregulation of β-catenin (A), loss of corresponding PTEN signal (B) and upregulation of $pAKT^{Ser473}$ (C) and $pmTOR^{Ser2448}$ (D). Table demonstrating proportions of cores that demonstrate combinations of up- and downregulation of B-catenin and PTEN (Upregulation is classified as ≥100 and downregulation as <100 using the histoscore technique) (E). Scatterplot demonstrating correlation between β-catenin and pAkt (F).

Each core size is 1.5mm

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