Activation of the PI3K/AKT Pathway Induces Urothelial Carcinoma of the Renal Pelvis: Identification in Human Tumors and Confirmation in Animal Models

Chao-Nan Qian,1,8 Kyle A. Furge,2 Jared Knol,1 Dan Huang,1 Jindong Chen,1 Karl J. Dykema,2 Eric J. Kort,3 Aaron Massie,1 Sok Kean Khoo,1 Kristin Vanden Beldt,4 James H. Resau,6 John Anema,7 Richard J. Kahnoski,1 Hans Morreau,9 Philippe Camparo,10 Eva Comperat,11 Mathilde Sibony,12 Yves Denoux,15 Vincent Molinie,13 Annick Vieillefond,14 Charis Eng,16 Bart O. Williams,5 and Bin Tean Teh1

Labsoratories of 1Cancer Genetics, 2Computational Biology, 3Molecular Epidemiology, 4Analytical, Cellular, and Molecular Microscopy, and 5Cell Signaling and Carcinogenesis and 6Division of Quantitative Sciences, Van Andel Research Institute; 7Department of Urology, Spectrum Health Hospital Grand Rapids, Michigan; 8State Key Laboratory of Oncology in Southern China, Sun Yat-sen University Cancer Center, Guangzhou, China; 9Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands; 10Service d’Anatomie Pathologique, Hôpital d’Instruction des Armées Val de Grâce; 11Service d’Anatomie Pathologique, CHU Pitié Salpêtrière; 12Service d’Anatomie Pathologique, CHU Ternon; 13Service d’Anatomie Pathologique, Hopital Saint Joseph; 14Service d’Anatomie Pathologique, CHU Cochin, Paris, France; 15Service d’Anatomie Pathologique, Hopital Foch, Suresnes, France; and 16Genomic Medicine Institute, Lerner Research Institute and Taussig Cancer Institute, Cleveland Clinic, Cleveland, Ohio

Abstract

Urothelial carcinoma of the renal pelvis is a deadly disease with an unclear tumorigenic mechanism. We conducted gene expression profiling on a set of human tumors of this type and identified a phosphatidylinositol 3-kinase (PI3K)/AKT activation expression signature in 76.9% (n = 13) of our samples. Sequence analysis found both activating mutations of PIK3CA (13.6%, n = 22) and loss of heterozygosity at the PTEN locus (25%, n = 8). In contrast, none of the other subtypes of kidney neoplasms (e.g., clear-cell renal cell carcinoma) harbored PIK3CA mutations (n = 87; P < 0.001). Immunohistochemical analysis of urothelial carcinoma samples found loss of PTEN protein expression (36.4%, n = 11) and elevation of phosphorylated mammalian target of rapamycin (mTOR; 63.6%, n = 11). To confirm the role of the PI3K/AKT pathway in urothelial carcinoma, we generated mice containing biallelic inactivation of Pten in the urogenital epithelia. These mice developed typical renal pelvic urothelial carcinomas, with an incidence of 57.1% in mice older than 1 year. Laser capture microdissection followed by PCR confirmed the deletion of Pten exons 4 and 5 in the animal tumor cells. Immunohistochemical analyses showed increased phosphorylated mTOR and phospho-S6K levels in the animal tumors. Renal lymph node metastases were found in 15.8% of the animals with urothelial carcinoma. In conclusion, we identified and confirmed an important role for the PI3K/AKT pathway in the development of urothelial carcinoma and suggested that inhibitors of this pathway (e.g., mTOR inhibitor) may serve as effective therapeutic agents.

Introduction

Urothelial carcinomas of the renal pelvis (also known as transitional cell carcinoma) account for 7% of all kidney tumors and ~5% of all urothelial tumors (1, 2). The standard treatment for this rare malignancy is open radical nephroureterectomy. However, endoscopic management is also reasonable in selected patients (3). The known prognostic factors include pathologic T stage, tumor grade, lymphovascular invasion, and tumor necrosis (1, 4–6). The proposed etiologic factors include smoking, exposure to occupational carcinogens, increased coffee consumption, and cyclophosphamide treatment (2). Although urothelial carcinoma is very common in families with Balkan nephropathy (7), the underlying molecular mechanism of tumorigenesis remains unclear. Interestingly, urothelial carcinoma is also a component tumor of Lynch syndrome [hereditary nonpolyposis colon cancer (HNPCC) syndrome], especially in the Muir-Torre variant (8, 9).

Phosphatidylinositol 3-kinase (PI3K) functions as a lipid kinase that catalyzes the formation of the second messenger, phosphatidylinositol-3,4,5-trisphosphate (PIP3), from phosphatidylinositol-4,5-bisphosphate (PIP2). PI3K-mediated production of PIP3 triggers a signaling cascade that results in the activation of the serine/threonine kinase AKT and some of its downstream targets, including p70 S6 kinase (S6K; refs. 10, 11). Functionally, the PI3K/AKT pathway phosphorylates and activates downstream targets, including mammalian target of rapamycin (mTOR). Activated mTOR itself phosphorylates and activates downstream targets, including p70 S6 kinase (S6K; refs. 10, 11). Functionally, the PI3K/AKT pathway can regulate numerous biological activities, including cellular growth, survival, and proliferation (12). Activating mutations in the PIK3CA p100 catalytic subunit α (PIK3CA) occur in ~30% of solid tumors (13–15). The majority of such mutations in PIK3CA occur in exons 20, 9, 1, and 7, in order of frequency (13, 15, 16).

The PTEN tumor suppressor functions primarily as a lipid phosphatase in the cytoplasm that converts PIP3 back to PIP2. By depleting the cellular levels of PIP3, PTEN acts as a brake on AKT activation. PTEN is commonly inactivated by mutation and loss of heterozygosity (LOH) in human cancers (17–20). Recently, nuclear PTEN has also been shown to play a fundamental role in the maintenance of chromosome stability, as well as a role

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Current address for S.K. Khoo: Laboratory of Microarray Technology, Division of Quantitative Sciences, Van Andel Research Institute, 333 Bostwick Avenue Northeast, Grand Rapids, MI 49503.

Requests for reprints: Chao-Nan Qian, Laboratory of Cancer Genetics, Van Andel Research Institute, 333 Bostwick Avenue Northeast, Grand Rapids, MI 49503. Phone: 616-990-5070; E-mail: chaonan.qian@vai.org or Bin Tean Teh, Laboratory of Cancer Genetics, Van Andel Research Institute, 333 Bostwick Avenue Northeast, Grand Rapids, MI 49503. Phone: 616-234-5296; Fax: 616-234-5297; E-mail: bin.teh@vai.org. ©2009 American Association for Cancer Research.

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in elicitng $G_1$ cell cycle arrest through its nuclear phosphatase activity (21, 22).

In our gene expression study evaluating different histologic subtypes of renal cell carcinoma (RCC), we found that renal pelvic urothelial carcinoma had a gene expression signature distinct from those of the clear-cell, papillary, chromophobe RCC/oncocytoma, and Wilms’ subtypes (23). We therefore hypothesized that the essential signaling pathway for the initiation of renal pelvic urothelial carcinoma could be identified by application of high-throughput screening technology and further confirmed by tissue-specific knockout animal models.

**Materials and Methods**

**Patient tissue samples.** Institutional review board approval was obtained from each participating institution providing human clinical samples. Frozen or formalin-fixed, paraffin-embedded tissue samples of 24 primary tumors with a diagnosis of urothelial carcinoma in the renal pelvis were collected from participating institutions in the United States (Spectrum Health Hospital, Grand Rapids, Michigan; Cooperative Human Tissue Network) and from the French Kidney Tumor Consortium. Of the 24 cases, 22 had sufficient tissues for DNA extraction, 13 had sufficient tissues for total RNA extraction, 11 had sufficient formalin-fixed, paraffin-embedded tissues for immunohistochemical (IHC) staining, and 8 had matched tumor/normal tissues appropriate for LOH analysis.

The DNA from another 87 kidney tumors of various pathologic types (32 clear-cell, 15 chromophobe, and 15 papillary RCC, 10 oncocytoma, 6 Wilms’ tumors, and 9 unknown kidney tumors) was also extracted for PIK3CA mutation screening. These kidney samples were obtained from the Cooperative Human Tissue Network with an approval from the Van Andel Research Institute Institutional Review Board.

**Gene expression analysis.** Gene expression profiles from 13 renal pelvic urothelial carcinoma samples were produced using the Affymetrix HG-U133 Plus 2.0 GeneChip platform, as described previously (24). Additional gene expression profiles derived from nondiseased kidney and from other subtypes of RCC were generated by our group and can be obtained from the Gene Expression Omnibus (GDS1344). Gene expression values were preprocessed using the RMA method as implemented in the BioConductor affy package for the R environment using updated probe set mappings (25). The gene expression data was filtered using an interquartile range filter (IQR >0.5) to identify the most variable genes ($n = 7,634$). Euclidian distance and complete linkage hierarchical clustering were used for unsupervised tumor sample evaluation. Pathway analysis was performed using a parametric gene-set enrichment analysis as implemented in the BioConductor PGSEA package (26). Briefly, sets of genes that were overexpressed or downexpressed by activation of MYC, RAS, E2F, SRC, AKT, synergistic HGF/vascular endothelial growth factor (VEGF); inactivation of VHL; or induction of hypoxia were obtained from the literature (27–31). HGF and VEGF signatures were generated using data from the Gene Expression Omnibus (GDS406 and GDS495, respectively). In all cases, cells after 24 h of treatment were compared with control cells. For consistent presentation, all “up” and “down” gene lists reflect the gene expression changes in treated/mutant cells versus the nearest approximation of wild-type cells; for example, MYC-transfected cells were compared with mock-transfected cells.

**Immunohistochemistry and semiquantitative analyses.** Rabbit monoclonal antibody (mAb) against PTEN (Abcam) was used for PTEN staining of mouse tissues and the human tissues using the Discovery XT System (Ventana Medical Systems, Inc.), which automatically prepared the IHC-stained slides, according to the manufacturer’s instructions. Mouse anti-PTEN mAb (1:50, Cell Signaling Technology, Inc.) was also used for manual staining of mouse tissues. Human anti-phosphorylated mTOR (1:20, Ser2448, Cell Signaling Technology, Inc.) and rabbit anti-phosphorylated S6-ribosomal protein (1:50, Ser240/244, Cell Signaling Technology, Inc.) were applied using the automatic staining system. Rabbit anti-phosphorylated AKT mAb (1:5, a gift from Katie Crosby, Cell Signaling Technology) was used for the manual staining of some human tissues. For manual staining, epitope retrieval was performed by heating the sections at 95°C in 10 mmol/L citrate buffer (pH 6.0) for 25 min. The sections were incubated with primary antibodies at 4°C overnight and visualized using a VECTASTAIN Elite ABC kit (POD; Vector Laboratories), 3,3-diaminobenzidine, and hematoxylin counterstaining. For semiquantitative analyses of the IHC staining of PTEN and phospho-mTOR, a scoring system was applied (32). Briefly, the IHC reaction was scored by multiplying

![Figure 1. Gene expression differences between urothelial carcinoma and other kidney tumors. Gene expression profiling data were obtained from nondiseased kidney cortex (Nor, n = 11), clear-cell (CC, n = 11), papillary type 1 (P1, n = 11), papillary type 2b (P2b, n = 6), and urothelial carcinomas or transitional cell carcinoma (TCC, n = 13) using the Affymetrix HG-U133 Plus 2.0 chipset. A, gene expression data were filtered using an IQR filter to identify the most variable genes (n = 7,634), and the samples were organized using Euclidian distance and complete linkage hierarchical clustering. B, the 50 most significantly upregulated and downregulated genes in the urothelial carcinoma samples were identified and the expression values were plotted as a heatmap (P < 1e-22). Red, the gene has increased expression in the urothelial carcinoma samples compared with the other samples. Blue, the gene has decreased expression.](image-url)
the percentage of positive tumor cells (PP: 0, no positive tumor cell; 1, <10%; 2, 10-50%; 3, 51-80%; 4, >80% positive tumor cells) by their prevalent degree of staining (SE: 0, negative; 1, weak; 2, moderate; 3, strong staining). Immunoreactive scores (IRS = PP × SI) range from 0 to 12. The average value from the scores of two independent observers was used as the final value. For phospho-mTOR staining, the interstitial tissues in all 11 cases were weakly stained with scores lower than 6. Therefore, we regard a score higher than 6 as indicating significant elevation of phospho-mTOR in the tumors.

**PIK3CA mutation screening.** Exons 1, 7, 9, and 20 of PIK3CA were amplified from genomic DNA with primers complimentary to surrounding intronic sequences (see Supplementary Table S2). PCR was carried out with 25 ng of genomic DNA in a reaction volume of 50 μL. Products were purified on Millipore MultiScreen HTS PCR plates, cycle-sequenced with BigDye v3.1 for 45 cycles at an annealing temperature of 55°C, and run on an ABI 3700 Genetic Analyzer.

**LOH analysis of the PTEN region.** Eight matched samples of renal pelvic urothelial carcinoma tumor tissues and normal tissues were studied using 12 highly polymorphic microsatellite markers from human chromosome 10: D10S1652, D10S537, D10S1686, W1218, D10S1739, W213, D10S1753, D10S564, D10S583, D10S185, D10S192, and D10S597. These markers are located from 10q21.2 to 10q25.1, flanking the PTEN gene (10q23.3). PCR was performed in a 7.5-μL reaction volume containing 0.17 μmol/L of each of fluorescence-labeled forward and unlabeled reverse primer, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 4 mmol/L MgCl2, 0.3 units each of fluorescence-labeled forward and unlabeled reverse primer, and 1 and 2 are the intensities of smaller and larger alleles, respectively. LOH was defined as the final value. For phospho-mTOR staining, the interstitial tissues in all 11 cases were weakly stained with scores lower than 6. Therefore, we regard a score higher than 6 as indicating significant elevation of phospho-mTOR in the tumors.

**Transgenic mice, genotyping, and histologic analyses.** Ksp-cadherin is a tissue-specific member of the cadherin family that is expressed exclusively in the epithelial cells of the kidney and the developing genitourinary tract (33). Ksp-Cre transgenic mice expressing Cre recombinase under the control of the Ksp-cadherin promoter have been established and used for organ-specific knockout studies (34). Pten−/− mice, carrying a pair of loxP sites that flank Pten exons 4 and 5 (35), were obtained from Tak Mak. We generated mice homozygous for the Pten−/− allele that also contained the Ksp-Cre transgene (Ksp-Cre/Pten−/−lox/lox). Genotyping was performed by PCR analysis of tail DNA. The primers used for Cre recombinase genotyping were as follows: 5′-CGATGCAACAGTGATAGTCTTC-3′ (CreF) and 5′-GCACAGTCAGCGCATCAAC-3′ (CreR). Pten−/− genotyping was carried out using the following primers: 5′-CATCACACTAAGGTCTGTGG-3′ (PtenF) and 5′-GTGAACCTCCCCACATAAAGC-3′ (Pten-11836R).

After euthanization of the mice, the studied organs and tissues were isolated and fixed in 10% neutral buffered formalin for routine H&E staining or IHC staining. All mouse experiments were approved before initiation by the Van Andel Institute Institutional Animal Care and Use Committee.

**Laser capture microdissection and PCR amplification.** Laser capture microdissection (LCM) was performed on the urothelial carcinoma tissues as previously reported (34). Briefly, sections (8 μm) were cut from the paraffin blocks and stained with H&E. LCM was then performed using a PixCell Ile LCM system (Arcturus Engineering, Inc.) following the manufacturer's protocols. Captured cells attached to the polymer film surface on the CapShur LCM caps (Arcturus Engineering, Inc.) were incubated with 150 μL of digestion buffer from PicoPure DNA extraction kit (Arcturus Engineering, Inc.) at 65°C for 24 h, followed by boiling for 10 min to inactivate the proteinase K.

Primers were designed to amplify the region flanked by loxP sites in the Pten−/− allele. A third primer was also used as indicated in Fig. 5C. Sequences were as follows: 5′-ATGATCTGTGATATCGTTCG-3′ (P1), 5′-TCACAGGGATTTAGACGATCC-3′ (P2), and 5′-AACAGACATCTGGAACCTTATCAG-3′ (P3). PCR was performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and 300 nmol/L of each primer. Four DNA samples were analyzed: (a) total urothelial carcinoma tumor tissues from a Ksp-Cre+/−/Pten−/−lox/lox mouse; (b) laser-captured urothelial carcinoma

Figure 2. Prediction of AKT activation from gene expression data. A, gene expression profiling data were analyzed using a parametric gene-set enrichment approach for evidence of deregulated signal transduction pathways. As described in Materials and Methods, for each signaling pathway, we generated lists of genes that exhibited altered expression in cells that were treated or mutated to affect that indicated pathway. For each pathway, the "up" gene signature is the list of genes that have increased expression in the treated/mutant cells relative to control cells (e.g., MYC-transfected versus mock-transfected cells). Likewise, the "down" signature indicates the set of genes that show decreased expression relative to control cells. The VHL gene signature was obtained from cells that contained inactivating VHL mutations. The resulting summary statistic (t statistic) for each gene list is plotted. Red, a significant number of genes in the list have increased expression in the tumor samples relative to the normal kidney. Blue, a significant number of genes in each list have decreased expression. Only the most significant data are displayed (P < 0.005). B and C, predictions of signal transduction pathway abnormalities were generated using the P-GSEA approach. In this approach, each sample receives a t statistic (t) and a significance value (P) that indicates that the gene signature is present in the tumor gene expression profile. t statistics generated from gene signatures indicative of AKT pathway activation and VHL pathway inactivation in the different tumor subtypes are shown. Dashed lines, samples that have the most significant gene-set enrichment (P < 0.005).
sight into the molecular genetic defects associated with these tumors. Genes that are overexpressed in urothelial carcinoma relative to normal kidney cortex and other kidney tumors were identified. The expression levels of several genes, e.g., S100P, Rab25, various keratins, and forkhead transcription factors, were consistent with previous gene expression profiling studies of urothelial carcinoma (37, 38). In agreement with its unique pathologic appearance, the gene expression profiling of urothelial carcinoma suggests that this carcinoma is a distinct subtype of kidney tumor (Fig. 1).

AKT pathway was prominently activated in urothelial carcinoma of the renal pelvis. The gene expression data was also examined for evidence of signal transduction defects using gene-set enrichment analysis (26, 27, 39, 40). Sets of genes that are regulated by known oncogenes and tumor suppressors were evaluated for deregulation in the urothelial carcinoma samples (Fig. 2A). This analysis revealed that a set of genes overexpressed following activation of PI3K/AKT in tissue culture cells was also significantly overexpressed in 10 of 13 urothelial carcinoma samples (76.9%; Fig. 2B). Clear-cell RCCs, which represent the majority of adult kidney tumors, are associated with biallelic inactivation of the VHL gene (41). Consistent with VHL inactivation, a set of VHL-regulated genes were significantly downregulated in the clear-cell RCC samples (Fig. 2C). The VHL-regulated genes were not significantly deregulated in the urothelial carcinoma samples, suggesting that defects in AKT signaling, but not VHL signaling, are associated with development of urothelial carcinoma of renal pelvis.

**PIK3CA mutations were found only in urothelial carcinoma of the renal pelvis.** Activating mutations in the catalytic subunit of PI3K (PIK3CA) are common occurrences in cancer. To determine if activating mutations in PIK3CA are associated with the predicted frequent activation of the PI3K/AKT pathway in renal pelvic urothelial carcinoma, sequence analysis of PIK3CA was performed on 22 human renal pelvic urothelial carcinomas and 87 cases of other types of renal tumors. Mutations of PIK3CA were found in 4 (18.2%) urothelial carcinoma cases (one tumor with K111E mutation in exon 1, two with E545K, and one with E542K in exon 9). Of these, the mutations of E545K and E542K in exon 9 occur in a hotspot of sequence mutation and are known activating mutations (13, 42–44). Therefore, at least a 13.6% (3 of 22) frequency of an activating PIK3CA mutation was found in the urothelial carcinoma samples (Supplementary Table S1). In contrast, no mutation was found in the 87 cases of other renal neoplasms; thus, the prevalence of activating mutations in PIK3CA is significantly higher in renal pelvic urothelial carcinoma ($P = 0.01$).

**LOH at the PTEN gene locus in urothelial carcinoma of the renal pelvis.** In addition to DNA sequence mutations, LOH of PTEN is a well-known event in many malignancies and serves to activate the PI3K/AKT pathway. To determine if LOH of PTEN occurs in renal pelvic urothelial carcinoma, we examined eight pairs of matched normal/tumor tissues. LOH at the PTEN gene locus was found in two cases, a 25% frequency (Supplementary Table S1).

**Loss of PTEN protein and elevation of phosphorylated mTOR in urothelial carcinoma of the renal pelvis.** To determine the protein levels of two important components of the PI3K/AKT pathway, PTEN and mTOR, IHC staining of human urothelial carcinoma of renal pelvis was performed, followed by semiquantitative scoring using a 0 to 12 scale system. Figure 3 shows an absence of PTEN protein in a renal pelvic urothelial carcinoma and elevated expression of phosphorylated AKT and phosphorylated mTOR. Of the 11 cases analyzed, 36.4% of the urothelial tissues from the same mouse; (c) tail DNA from the same mouse; and (d) tail DNA from a mouse homozygous for the wild-type Pten allele.

**Statistical analyses.** Genes differentially expressed between urothelial carcinoma and the other tumor types were identified using a moderated t statistic as implemented in the limma BioConductor/R package (36). Significance values were adjusted using the false discovery rate method to compensate for multiple testing. A two-sided Student’s t test was used to determine if the expression of the genes associated with pathway activation or repression were deregulated in each individual tumor sample when compared with the median expression of the same genes in the nondiseased samples. A χ² test was used to compare the incidence rates of predicted PI3K/AKT activation from gene expression data. A Fisher’s exact test was used for comparison of the somatic activating mutation rates of PIK3CA between TCC and other types of kidney tumors.

**Results**

**Unique gene expression profile was revealed in human urothelial carcinoma of the renal pelvis.** Gene expression profiling was performed on a set of renal urothelial carcinomas to gain insight into the molecular genetic defects associated with these tumors. Genes that are overexpressed in urothelial carcinoma relative to normal kidney cortex and other kidney tumors were identified. The expression levels of several genes, e.g., S100P, Rab25, various keratins, and forkhead transcription factors, were consistent with previous gene expression profiling studies of urothelial carcinoma (37, 38). In agreement with its unique pathologic appearance, the gene expression profiling of urothelial carcinoma suggests that this carcinoma is a distinct subtype of kidney tumor (Fig. 1).

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carcinomas had loss of PTEN expression and 63.6% had elevation of phosphorylated mTOR (Supplementary Table S1).

**Mice with homozygous Pten deletion harbored typical renal pelvic urothelial carcinomas.** To verify the role of the PI3K/AKT pathway in the tumorigenesis of human renal pelvic urothelial carcinoma, mice were generated that carried a conditional deletion of the Pten gene specifically in the renal epithelium, using the Ksp-Cre/lox system. Whereas neither transitional epithelial hyperplasia nor urothelial carcinoma was found in wild-type or monoallelic Pten knockout (Ksp-Cre/Pten^flox/+^) mice, typical urothelial carcinomas of renal pelvis were found in homozygous Pten deletion (Ksp-Cre/Pten^flox/flox^) mice (Fig. 4). It was common in homozygous Pten deletion mice older than 1 year that urothelial carcinoma involving the uretero-pelvic junction obstructed urine outflow and caused hydronephrosis (Fig. 4A). Moreover, urothelial carcinoma in this animal model invaded through the muscular layer of the renal pelvis and into the surrounding fat tissue (Fig. 4C). Importantly, renal lymph node metastases were also found in 15.8% of the animals with urothelial carcinoma (Fig. 4B). The incidence rates of urothelial carcinoma of the renal pelvis and precancerous transitional epithelial hyperplasia in homozygous Pten deletion mice increased with age (Fig. 5A). The incidence of renal pelvic urothelial carcinoma was 18.2% in mice younger than 6 months and increased to 57.1% in mice older than 1 year.

To confirm the inactivation of Pten in murine urothelial carcinomas, tissues were isolated using LCM (Fig. 5B) followed by PCR analysis to characterize the Pten locus in the tumors (Fig. 5C). This analysis confirmed the deletion of Pten exons 4 and 5 in the mouse urothelial carcinoma tissues (Fig. 5D).

**Activation of the Akt pathway in mouse urothelial carcinomas of the renal pelvis.** IHC staining of PTEN, mTOR, and S6K (a downstream kinase regulated by mTOR) was performed on murine urothelial carcinoma tumor sections to determine the status of these proteins. Figure 6A clearly shows that the absence of Pten was accompanied by elevated expression of phosphorylated mTOR and phosphorylated S6K.

**Polycystic renal dysplasia and genital carcinomas following Akt pathway activation.** Because the Ksp-cadherin promoter is expressed in the epithelial cells of the kidney as well as the developing genitourinary tract (33), inactivation of Pten in our animal model also resulted in some structural abnormalities in the renal parenchyma and the genital organs.

Polycystic tubular abnormalities, consisting chiefly of simple tubular cysts that were lined by a single layer of epithelial cells (Fig. 4D, left), occurred in all kidneys of homozygous Pten deletion mice in every age group (Fig. 5A). This abnormality was present in 50% or less of the kidneys of heterozygous Pten deletion mice in every age group and increased in frequency as.
the animals aged. The consistency of the absence of Pten protein and the presence of phosphorylated S6K protein (Fig. 6B and C) in these tubular epithelial cells confirmed the activation of Akt pathway in this alteration.

Hyperplasia of tubular epithelia in the renal parenchyma (Fig. 4D, right) only occurred in the homozygous Pten deletion mice, and the frequency also increased with age (Fig. 5A, right). Systemic homozygous inactivation of Pten results in early embryonic death (45, 46). It has been reported that systemic heterozygous inactivation of Pten induces neoplasms in multiple organs, including the endometrium (45, 47). Consistent with these findings, endometrial carcinomas and seminal vesicle carcinomas were found in our study in 43.5% of the homozygous Pten deletion mice (Fig. 4A) and were associated with elevated expression of phosphorylated mTOR in the tumor cells (Supplementary Fig. S1).

Discussion

We conducted a comprehensive study to elucidate the role of the PI3K/AKT pathway activation in the development of renal pelvic urothelial carcinoma. We first identified differentially expressed genes consistent with the activation of PI3K/AKT pathway in human renal pelvic urothelial carcinoma using high-throughput gene expression profiling. Subsequently, we found that 13.6% of these human tumors contained activating somatic PIK3CA mutations and 25% had LOH in and around the PTEN locus. In addition, 54.5% of these human urothelial carcinomas had significantly decreased or absent expression of PTEN protein, whereas 100% displayed increased phospho-mTOR expression. These data all support a key role for the PI3K/AKT pathway in human renal pelvic urothelial carcinoma. Finally, we were able to show induction of renal pelvic urothelial carcinoma highly similar to that of humans by means of a homozygous tissue-specific Pten
deletion and activation of Akt and mTOR signaling in a murine model.

Patients with upper-tract urothelial carcinoma are usually elderly. A study involving >5,000 patients between 1985 and 1996 placed the mean age of urothelial carcinoma development as 70 years old (48). Consistent with this observation, our renal-specific Pten knockout mice exhibit increasing prevalence of renal pelvic urothelial carcinoma with age, from 18.2% when younger than 6 months to 57.1% when older than 12 months (Fig. 5A, left). The late occurrence of renal pelvic urothelial carcinoma in both human and animal models implies that genetic or environmental factors, in addition to PI3K/AKT pathway activation, may be involved in the initiation of renal urothelial carcinoma. Our mouse model may be a unique tool for addressing this issue.

The identification of AKT pathway activation in urothelial carcinoma suggests that targeting this kinase or its targets could provide therapeutic benefits for the majority of patients with this deadly disease. It has been reported that the members of HNPCC families have a 14-fold greater risk of developing urothelial carcinoma relative to the general population with the same ethnic background (8). HNPCC is caused by germline mutations in the mismatch repair genes. Mismatch repair deficiency in this setting results in the cellular phenotype known as microsatellite instability, which particularly affects mononucleotide repeat tracts. In subsets of HNPCC-related colorectal cancers and endometrial cancers, somatic mutations targeting the 6A tracts in exons 7 and 8 of PTEN have been found, resulting in upregulation of the AKT pathway (9). Therefore, based on these and our current report, we propose that intervention against AKT, or toward downstream targets such as mTOR, might also be an effective cancer prevention approach for individuals in HNPCC families. For example, rapamycin and its analogues are currently being tested in clinical trials in a variety of settings and could be rapidly integrated into the treatment of renal pelvic urothelial carcinoma.

To our knowledge, our animal model is the first model that can generate spontaneous renal pelvic urothelial carcinoma. Interestingly, urothelial carcinoma of the renal pelvis accompanied by autosomal dominant polycystic kidney disease has been reported, but with an unclear molecular mechanism (49, 50). In our conditional Pten knockout mice, renal pelvic urothelial carcinoma was commonly accompanied by polycystic renal dysplasia, Figure 6.

**Figure 6.** Activation of the Akt pathway in murine renal pelvic urothelial carcinoma and polycystic renal dysplasia following inactivation of Pten. A, continuous sections of a murine renal pelvic urothelial carcinoma are routinely stained with H&E as well as IHC stained with anti-PTEN, anti-phospho-mTOR, and anti-phospho-S6K antibodies. The absence of PTEN in the tumor is accompanied by elevated expression of phospho-mTOR and accumulation of phospho-S6. B, continuous sections of a kidney from a homozygous Pten deletion were stained with anti-PTEN antibody (left) and anti-phospho-S6K antibody (right). Note that the absence of PTEN is consistent with the presence of phospho-S6K in the epithelium of a renal cyst (star), indicating the activation of Akt pathway in the involved epithelial cells. C, continuous sections of a kidney from a homozygous Pten deletion mouse were stained with anti-PTEN antibody (left) and anti-phospho-S6K antibody (right). Note that the mosaic absence of PTEN in two renal tubules (white arrows, left) is consistent with the mosaic presence of phospho-S6K in these tubules (black arrows, right).
mimicking the clinical manifestations of this human disease. Therefore, our model could be very useful in fully elucidating the related molecular mechanism(s) of urothelial carcinoma with polycystic kidney disease.

A pathologic similarity has been observed between urothelial carcinoma of upper tract and bladder urothelial carcinoma, but the patterns of disease relapse are very different. Bladder urothelial carcinoma occurs in 15% to 50% of patients following urothelial carcinoma of upper tract, whereas the latter only occurs in 2% to 4% of bladder urothelial carcinoma patients with a longer relapse-free survival (3). In our study, no alteration of the transition epithelium in the bladder was observed, although Cre recombination has been reported to be expressed in the developing genitourinary tract of Ksp-Cre transgenic mice (33). This implies that important differences in tumorigenic susceptibility exist in different parts of the transitional epithelia that line the urinary tract.

In summary, this study reports a comprehensive translational study involving a high-throughput screening technology and the development of an animal model to systematically identify a uniquely activated molecular pathway in urothelial carcinoma of the renal pelvis and to further confirm the carcinogenic role of this pathway in the initiation of the malignancy. We found that the AKT pathway was activated in the majority of human renal pelvic urothelial carcinomas, which might be partly due to activating mutations of PIK3CA and the loss of PTEN. Conditional knockout of the Pten gene results in renal pelvic urothelial carcinoma in mice, which confirms the etiologic effect of AKT pathway activation in this malignancy. Importantly, our report implicating the upregulation of AKT and downstream effectors such as mTOR in the development of urothelial carcinoma lends support to the testing of mTOR inhibitors in treating human renal pelvis urothelial carcinoma in various settings, both sporadic and heritable.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Chao-Nan Qian, Kyle A. Furge, Jared Knol, et al.


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