Inactivation of p53 and Pten promotes invasive bladder cancer

Anna M. Puzio-Kuter,1,2,3 Mireia Castillo-Martín,1,2,4 Carolyn W. Kinkade,1,2,3 Xi Wang,2,5,6 Tian Huai Shen,2,4 Tulio Matos,2,4 Michael M. Shen,2,5,6 Carlos Cordon-Cardo,1,2,4,8 and Cory Abate-Shen1,2,3,4,7

1Department of Urology, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA; 2Herbert Irving Comprehensive Cancer Center, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA; 3Center for Advanced Biotechnology and Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, USA; 4Department of Pathology, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA; 5Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA; 6Department of Genetics and Development, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA

Although bladder cancer represents a serious health problem worldwide, relevant mouse models for investigating disease progression or therapeutic targets have been lacking. We show that combined deletion of p53 and Pten in bladder epithelium leads to invasive cancer in a novel mouse model. Inactivation of p53 and PTEN promotes tumorigenesis in human bladder cells and is correlated with poor survival in human tumors. Furthermore, the synergistic effects of p53 and Pten deletion are mediated by deregulation of mammalian target of rapamycin (mTOR) signaling, consistent with the ability of rapamycin to block bladder tumorigenesis in preclinical studies. Our integrated analyses of mouse and human bladder cancer provide a rationale for investigating mTOR inhibition for treatment of patients with invasive disease.

Supplemental material is available at http://www.genesdev.org.

Received December 15, 2008; revised version accepted January 30, 2009.

Bladder cancer is the fifth most common malignancy occurring worldwide and a major cause of cancer morbidity and mortality (Jemal et al. 2005). Approximately 90% of bladder tumors are of epithelial origin, the majority corresponding to transitional cell carcinomas (Dinney et al. 2004; Eble et al. 2004; Reuter 2006). Early stage bladder tumors comprise two groups: low-grade, which are always papillary and usually superficial, and high-grade carcinoma in situ (CIS), which is the precursor of invasive bladder cancer. Superficial tumors, which account for the large majority (~75%–85%), typically have a favorable prognosis, while the 5-yr survival for patients with invasive bladder cancer (~25% of all bladder tumors) can be less than 10% (Jemal et al. 2005). Notably, superficial and invasive bladder cancers are associated with distinct genotypic and phenotypic patterns [Wu 2005; Cordon-Cardo 2008; Knowles 2008]. In particular, chromosome 9 deletions and mutations of RAS and FGFR3 occur in most, if not all, superficial papillary noninvasive tumors, but only in a small subset of invasive bladder tumors. In contrast, deletions of 3p, 5q, 10q (PTEN locus), 11p, 13q (RB locus), 17p (TP53 locus), and 18q (DCC locus) are absent or at least rare in superficial tumors, but occur frequently in invasive bladder carcinomas.

Until now, there has been a paucity of model systems that recapitulate invasive bladder cancer and thereby facilitate analyses of pathways of disease progression or identification and evaluation of targets for therapeutic intervention. Here, we describe a new mouse model of invasive bladder cancer that recapitulates many aspects of human bladder cancer. By integrating analyses from this new mouse model with correlative and functional data from human bladder cancer, we show that combinatorial inactivation of p53 and Pten are major causal factors that predict poor outcome of invasive bladder cancer. We further demonstrate that inactivation of p53 and Pten leads to deregulation of the mammalian target of rapamycin (mTOR) signaling pathway, and, consequently, that inhibition of this signaling pathway blocks bladder tumor growth. Our findings provide a relevant preclinical model for therapeutic investigations, as well as a strong rationale for targeting the mTOR signaling pathway in patients with invasive bladder cancer.

Results and Discussion

A new mouse model of invasive bladder cancer

The bladder epithelium is comprised of several different cell types, including umbrella cells that line the bladder lumen, intermediate cells, and basal cells, which are adjacent to the lamina propria (Fig. 1A). Since the relationships of these cell types for bladder tumorigenesis has not yet been resolved, we utilized an approach for gene deletion that is not targeted to a particular cell type and/or differentiation status in the bladder epithelium. Specifically, we surgically delivered an adenovirus expressing Cre recombinase (hereafter referred to as Adeno-Cre) into the bladder lumen of adult male mice to induce conditional gene deletion in the epithelium (Fig. 1A). Using an R26R reporter allele to evaluate targeting efficiency and specificity, we found that Adeno-Cre resulted in sporadic (~10%) gene deletion exclusively in the epithelium and not in the underlying lamina propria or muscle layers [n = 10] (Fig. 1B,C).

We utilized this approach to assess the functional consequences of deleting tumor suppressor genes using the appropriate conditional alleles. Since inactivation of p53 leads to bladder tumors in transgenic mice [Zhang et al. 1999], and Pten deletion results in hyperplasia of
Targeted gene deletion in bladder epithelium via Adeno-Cre delivery. (A) Schematic of bladder anatomy showing the layers of the epithelium. Adeno-Cre (1×10^10 PFU) was delivered into the bladder lumen of R26R reporter mice and the location and extent of recombination was analyzed 3 d later by visualization of β-galactosidase staining [B] or by immunofluorescence with colabeling by cytokeratin 7 to mark epithelial cells [C].

Figure 1. Targeted gene deletion in bladder epithelium via Adeno-Cre delivery. (A) Schematic of bladder anatomy showing the layers of the epithelium. Adeno-Cre (1×10^10 PFU) was delivered into the bladder lumen of R26R reporter mice and the location and extent of recombination was analyzed 3 d later by visualization of β-galactosidase staining [B] or by immunofluorescence with colabeling by cytokeratin 7 to mark epithelial cells [C].

Next, we investigated the relevance of p53 and PTEN inactivation with outcome or the coordinated alteration of any that have investigated the association of PTEN status with outcome (Esrig et al. 1994; Lianes et al. 1994; Sarkis et al. 1994) and have examined PTEN status (Cairns et al. 1998; Aveyard et al. 1999, Tsuruta et al. 2006), we are not aware of any that have investigated the association of PTEN inactivation with outcome or the coordinated alteration of p53 and PTEN in human bladder cancer. Therefore, we examined p53 and PTEN expression in tissue microarrays (TMAs) from two independent cohorts of human bladder tumors. The first had a total of 165 cases, including 135 cases of transitional cell carcinomas representative of all histological grades and stages, while the second included 85 cases from patients with muscle-invasive bladder cancer with extensive clinical follow-up data.

As expected, p53 was wild-type [undetectable] in non-invasive papillary tumors (n = 23), while p53 alterations either p53 or Pten [p53^fl/fl, Pten^fl/fl, Rb^fl/fl, or Pten^fl/fl, Rb^fl/fl] did not produce bladder tumors or result in grossly abnormal bladder histology (Table 1, Supplemental Fig. 6). We conclude that this Adeno-Cre delivery approach demonstrates a specific requirement for deletion of p53 and Pten for invasive bladder tumors in mice.

Figure 2. p53 and Pten collaborate in suppression of invasive bladder cancer in mutant mice. (A) Survival curve for mice of the indicated genotypes injected with Adeno-Cre and monitored for the first occurrence of invasive bladder tumors for up to 1yr following delivery of Adeno-Cre, while the histology of their bladder epithelium was within normal limits [Fig. 2A; Table 1; Supplemental Fig. 5]. Furthermore, the p53; Pten compound mice contrast with mice harboring mutations of Rb, which has been implicated in bladder tumorigenesis (Cordon-Cardo 2008; Knowles 2008), since deletion of Rb in bladder epithelium alone or in combination with
p53 nuclear overexpression and/or TP53 gene mutations) were frequently observed in invasive bladder tumors \( (n = 54) \) (Fig. 3A–D). Conversely, while PTEN was uniformly expressed in the epithelium of noninvasive papillary tumors \( (pT\text{A}, n = 23) \), it was frequently down-regulated in invasive bladder tumors \( (37 \text{ of } 53 \text{ pT1-pT2, } 17 \text{ of } 78 \text{ pT3-4 lesions; } P < 0.05) \) (Fig. 3E,F). Importantly, a high percentage of human bladder tumors with altered p53 also had deregulated expression of PTEN \( (41\%, n = 67) \) (see Fig. 3D,F).

Multivariate analyses revealed that altered expression of PTEN was associated with adverse patient outcome \( (P = 0.005) \) and suggested that their combinatorial alteration in human bladder tumors defines a subgroup of patients with a particularly aggressive clinical course.

**Preclinical analyses of targeted pathways for invasive bladder cancer**

In other tissue contexts, cooperativity of Pten and p53 has been shown to reflect their cross-talk on convergent signaling pathways, particularly involving mTOR signaling [Feng et al. 2005; Cully et al. 2006]. We found that this pathway was also deregulated in mouse and human bladder epithelial cell lines that have both p53 and PTEN intact [Sanchez-Carbayo et al. 2002].

While the control RT4 cell recombinants formed low-grade uroepithelial tumor lesions \( (n = 4) \), cell recombinants with RT4 cells in which either p53 or PTEN alone was knocked down resembled the control RT4 cell recombinants \( (n = 8 \text{ per group}) \) (Supplemental Fig. 7; Supplemental Table 1). Together with the correlative data in human bladder tumors and analyses of the mutant mice, these data demonstrate that p53 and PTEN functionally cooperate in suppression of bladder cancer and suggest that their combinatorial alteration in human bladder tumors defines a subgroup of patients with a particularly aggressive clinical course.

**Table 1: Summary of the phenotype of the mutant mouse model**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mths post allograft</th>
<th>N</th>
<th>Bladder weight (mg)</th>
<th>Metastases</th>
<th>Bladder phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53+/−; Pten+/−</td>
<td>Up to 14</td>
<td>21</td>
<td>38.07±6.427 (N=18)</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>p53−/−; Pten−/−</td>
<td>Up to 14</td>
<td>29</td>
<td>37.80μg±5.590 (N=22)</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>p53−/−; Pten+/−</td>
<td>Up to 14</td>
<td>31</td>
<td>43.23μg±0.535 (N=25)</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>p53+/−; Pten−/−</td>
<td>Up to 8</td>
<td>9</td>
<td>44.64μg±0.567 (N=0)</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>p53+/−; Pten+/−</td>
<td>Up to 8</td>
<td>7</td>
<td>38.85μg±1.044 (N=0)</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>p53+/−; Pten+/− (females)</td>
<td>0 to 2.5</td>
<td>15</td>
<td>139.75±1.079 (N=19)</td>
<td>None</td>
<td>Small tumors: In situ/CIS</td>
</tr>
<tr>
<td>p53+/−; Pten+/− (females)</td>
<td>2.5 to 6</td>
<td>15</td>
<td>157.95±1.186 (N=15)</td>
<td>2/10 (20%)</td>
<td>Large tumors: CIS and invasion</td>
</tr>
<tr>
<td>p53+/−; Pten+/− (females)</td>
<td>4.0 to 6</td>
<td>14</td>
<td>24/03±1.286 (N=15)</td>
<td>6/10 (60%)</td>
<td>Large tumors: CIS, invasion, and visible metastases</td>
</tr>
<tr>
<td>p30−/−; Pten−/−</td>
<td>Up to 6</td>
<td>5</td>
<td>52/74±13/72±141 (N=6)</td>
<td>5/4 (75%)</td>
<td>Large tumors: CIS and invasion</td>
</tr>
<tr>
<td>p30−/−; Pten−/−</td>
<td>Up to 12</td>
<td>13</td>
<td>26.83μg±1.001 (N=13)</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>p53+/−; Pten−/−</td>
<td>Up to 12</td>
<td>4</td>
<td>33.40±2.037 (N=4)</td>
<td>None</td>
<td>Normal</td>
</tr>
</tbody>
</table>

1. At least 50% of the mice in each group had the R26R.GFP reporter allele for verification of targeting to bladder epithelium.
2. The total number \( (N) \) in each group includes mice found dead and/or otherwise not subject to complete phenotypic and histological analysis.
3. Refers to visible metastases \( (\text{Mets}) \) to lymph nodes, spleen, liver, and diaphragm.

**Figure 3.** Altered p53 and PTEN are associated with poor survival in human bladder cancer. (A–F) Tissue microarray analyses of p53 and PTEN showing immunohistochemical staining of human bladder tumors \( (P < 0.001) \) (Fig. 3G). Moreover, patients with tumors classified as having alterations of both p53 and PTEN had a median survival of 6 mo versus those having p53 mutations but normal PTEN expression, who had a median survival 6 yr \( (P < 0.001) \) (Fig. 3H). Therefore, combined alterations of p53 and PTEN occur frequently in invasive bladder cancer and are associated with poor patient outcome.
of bladder weights. (Supplemental Fig. 8A–D). Furthermore, the mouse and human bladder tumors from mutant mice. (Fig. 4F–O, Supplemental Fig. 8E–N). For the prevention paradigm, beginning 1 wk following Adeno-Cre injection, we delivered vehicle or rapamycin [10 mg/kg], an inhibitor of mTORC1 (Bjornsti and Houghton 2004; Guertin and Sabatini 2007), for up to 5 mo and examined the consequences for bladder tumorigenesis. While the vehicle-treated mutant mice developed large bladder tumors as expected [2241.55 ± 257mg], the bladders from the rapamycin-treated mice appeared grossly normal, having normal bladder weights [28.7 ± 0.782 mg] and no obvious histological abnormalities (n = 5 per group) [Fig. 4F–I,N]. Furthermore, the bladder epithelium from rapamycin-treated mice displayed 16-fold reduced cellular proliferation relative to vehicle-treated counterparts (P < 0.0001) and little or no expression of p-S6, a read-out of the mTOR signaling pathway [Fig. 4J–M,O]. Therefore, rapamycin was highly effective for prevention of bladder tumor growth in the p53flox/flox, Ptenflox/flox mutant mice.

To complement these analyses, we investigated the consequences of rapamycin in a treatment paradigm, namely, the effect on growth of established tumors in a tissue recombination/renal grafting model. Specifically, cells from dissociated mouse bladder tumors were recombined with embryonic bladder mesenchymal cells, followed by growth for three months as renal grafts in vivo, during which time the tumor-bearing mice were treated daily with rapamycin or vehicle via i.p. injection [Supplemental Fig. 8E–N]. The rapamycin-treated grafts displayed a significant reduction in tumor size [28 ± 5mg, n = 17] relative to vehicle-treated grafts [151 ± 16mg, n = 20], coincident with a significant reduction in cellular proliferation [P < 0.0001] and reduced expression of p-S6 [Supplemental Fig. 8E–N]. Thus, these preclinical studies demonstrate the efficacy of rapamycin as an inhibitor of bladder tumor growth in vivo.

Conclusions

Mutations of p53 or PTEN are among the most frequent causal events in many cancers, and their combined inactivation has profound consequences for tumorigenesis in numerous contexts, including lymphoma, prostate cancer, glioblastoma, and medulloblastoma [Mao et al. 2003; Chen et al. 2005; Cully et al. 2006; Hambardzumyan et al. 2008; Kwon et al. 2008; Zheng et al. 2008]. Our current findings establish the relevance of combinatorial inactivation of p53 and PTEN for invasive bladder cancer and provide new insights regarding their context-dependent functions in tumor progression. Indeed, while a primary role for p53 in invasive bladder cancer has been well established [Cordon-Cardo 2008;
Materials and methods

Mouse alleles were obtained from the NCI Mouse models repository or the Jackson Laboratories. Adenovirus expressing Cre recombinase was obtained from University of Iowa’s Vector Core Facility and was delivered to the bladder lumen. At the time of sacrifice, tissues were collected and processed for histological, immunohistochemical, immunofluorescence, or Western blot analyses, as detailed in the Supplemental Material. Details of all antibodies are provided in the Supplemental Material.

Cell recombination assays were done by combining RT4 cells with rat embryonic mesenchyme and grown under the renal capsule of nude male mice. Tissue recombinants were made using normal bladder epithelium or tumors from mouse, recombined with rat embryonic mesenchyme and grown in culture or in the renal capsule of nude male mice. Knockdown studies were done with lentiviral RNAi from Sigma-Aldrich’s Mission shRNA library (Sigma-Aldrich). Details of all lentiviral constructs are provided in the Supplemental Material. Rapamycin (from LC Laboratories) was provided once daily via i.p. at 10 mg/kg in vivo for up to 5 mo.

Human bladder cancer TMAs were made from a patient cohort of 165 tumors, with 136 transitional cell carcinomas and a second cohort of 86 muscle-invasive bladder cancers with clinical follow-up data. Scoring of the TMAs took into account the percentage of immunopositive tumor cells and intensity of staining. Statistical analyses were done using Mann-Whitney U-test, the \( \chi^2 \) test, or Fisher’s exact test, and the Spearman correlation. Survival analysis was conducted by the log rank test and the Cox proportional hazard model.

Acknowledgments

This work was supported by grants U01 CA084294 (C.A.-S.), CA115985 (M.M.S.), P30 CA13696 (C.C.-C.), NCI P01 CA87497 (C.C.-C.), and NCI P50 CA91846 (C.C.-C.); the TJ Martell Foundation (C.A.-S. and C.C.-C.); and a Ruth L. Kirschstein National Research Service Award (CA1106625) to A.P.-K. M.M.S. and C.A.-S. are members of the NCI Mouse Models of Human Cancer Consortium.

References


