Hyperplasia and Carcinomas in Pten-Deficient Mice and Reduced PTEN Protein in Human Bladder Cancer Patients

Hirosi Tsuruta,1,2 Hiroyuki Kishimoto,2 Takehiko Sasaki,3 Yasuo Horie,4 Miyuki Natsui,2 Yoshiko Shibata,1 Koichi Hamada,1 Nobuyuki Yajima,1 Koichi Kawahara,1 Masato Sasaki,1 Norihiko Tsuchiya,1 Katsuhiko Enomoto,1 Tak Wah Mak,2 Toru Nakano,7 Tomonori Habuchi,1 and Akira Suzuki2

Departments of Urology,4 Molecular Biology,5 Microbiology,6 Gastroenterology, and Pathology, Akita University School of Medicine, Akita, Japan;1 The Campbell Family Institute for Breast Cancer Research, and Departments of Immunology and Medical Biophysics, University of Toronto, Toronto, Ontario, Canada; and2 Department of Pathology, Medical School and Graduate School of Frontier Biosciences, Osaka University, Suita, Japan

Abstract
PTEN is a tumor suppressor gene mutated in many human cancers. We used the Cre-loxP system to generate an urothelium-specific null mutation of Pten in mice [FabpCrePtenfloxflox (FPtenfloxflox) mice]. Histologic examination revealed that all FPtenfloxflox mice exhibited urothelial hyperplasia in which component cells showed enlarged nuclei and increased cell size. With time, 10% of FPtenfloxflox mice spontaneously developed pedicellate papillary transitional cell carcinomas (TCC). This type of tumor also arose in FPtenfloxflox mice treated with the chemical carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine. FPtenfloxflox urothelial cells were hyperproliferative and showed increased activation of the survival signaling molecules Akt and extracellular signal-regulated kinase. In humans, 53% of primary bladder cancer patients exhibited decreased or absent expression of PTEN protein in either the cytoplasm or nucleus of tumor cells. In early bladder cancers, PTEN expression was repressed in 42% of superficial papillary TCC but in only 8% of cases of carcinoma in situ (CIS). In advanced bladder cancers, PTEN protein was significantly reduced (particularly in the nucleus) in 94% of cases, and this decrease in PTEN correlated with disease stage and grade. Thus, PTEN deficiency may contribute to bladder cancer both by initiating superficial papillary TCC and by promoting the progression of CIS to invasive and metastatic forms. (Cancer Res 2006; 66(17): 8389-96)

Introduction
Bladder cancer is the fifth most common malignancy in the United States, and 95% of these tumors are transitional urothelial cell carcinomas (TCC). Two variants of TCC exist: superficial papillary-type TCC and nonpapillary nodular-type TCC. Superficial papillary TCC, which account for 70% to 80% of all urothelial tumors, present as superficial papillary lesions that are often multifocal and recurrent but only infrequently invade the underlying muscle (1). The 5-year survival rate of this variant (when treated promptly) approaches 90%. However, nonpapillary nodular TCC, which account for 20% to 30% of urothelial malignancies, are invasive at diagnosis and carry a very high risk of further invasion and metastasis. At least 50% of patients with muscle-invasive tumors will die within 2 years of diagnosis (2). Nonpapillary nodular type TCC are believed to develop from carcinoma in situ (CIS), early-stage urothelial tumors of highly malignant potential. Different genetic defects may underlie these bladder cancer variants. Activating mutations of fibroblast growth factor receptor 3 (FGFR3) are frequently (>70%) found in superficial papillary TCC, whereas dysfunction of p53 or Rb is associated with CIS and nonpapillary nodular TCC (3–5).

Mutations of PTEN occur in many human sporadic cancers and in hereditary tumor susceptibility disorders, such as Cowden’s disease (6). PTEN is a multifunctional phosphatase whose major substrate is phosphatidylinositol-3,4,5-triphosphate (PIP3; ref. 7), a lipid second messenger molecule. PIP3 is generated by the action of phosphatidylinositol 3-kinase (PI3K) that become activated by growth factors or hormones (8). PIP3 in turn activates numerous downstream targets, including the serine/threonine kinase Akt/protein kinase B involved in antiapoptosis, proliferation, and oncogenesis (9). By using its lipid phosphatase activity to dephosphorylate PIP3 at the cell membrane, PTEN negatively regulates the PI3K/Akt pathway and exerts tumor suppression. PTEN can also dephosphorylate FAK and Shc, activating the extracellular signal-regulated kinase (ERK) pathway (10). Whereas the functions of PTEN at the cell membrane are reasonably well understood, the roles of PTEN and PI3K in the nucleus are less clear. Several lines of evidence point to an additional tumor suppressive role for PTEN in the nucleus. (a) Nucleus-specific expression of PTEN reduces cell proliferation dependent on nuclear PIP3 (11). (b) A nuclear PIP3 receptor is involved in the inhibition of apoptosis (12). (c) PTEN affects the function of nuclear p53 directly and indirectly (13). (d) PTEN binds to and negatively regulates MSP58, a nuclear molecule capable of cell transformation (14).

It remains unclear whether PTEN deficiency contributes to the onset or progression of bladder tumors in vivo. In mice heterozygous for a null Pten mutation, bladder cancers are not generally observed (15). In humans, mutation or deletion of PTEN DNA occurs at a low frequency (0-32%) in primary bladder cancers and bladder cancer cell lines (~ 30%; refs. 16–19), but the status of PTEN protein remains to be definitively investigated. Akt is activated in 55% of primary bladder cancers (20), and PTEN overexpression induces growth suppression and increased sensitivity to doxorubicin in bladder cancer cells in vivo (21). Moreover, inhibitors of PTEN or PI3K reduce the motility and invasiveness...
of these cells (20). However, the role of PTEN in human primary bladder cancers remains to be fully elucidated.

We previously generated conditional mutant mice lacking Pten expression in various tissues and showed that Pten deficiency is usually associated with Akt and ERK activation, enlarged cell size, and tissue hyperplasia with tumor formation (22). Here, we show that mice deficient for Pten in the urothelium [FabpCrePten$^{lox/lox}$ mice] exhibit bladder cell hyperplasia and carcinomas. PTEN may therefore be an important regulator of bladder cancer initiation and/or progression.

Materials and Methods

Generation of FPTen$^{lox/lox}$ mice. Pten$^{lox/lox}$ mice (1290a × C57BL6 F6 background), generated as described previously (23), were mated to FabpCre transgenic mice (FVB/N × C57BL6 F4 background) in which Cre expression is controlled by the fatty acid–binding protein promoter (24). FabpCre directs recombination in all cell layers of the transitional epithelium that lines the renal calyces and pelvis, ureters, and bladder by embryonic day 16.5 (24). Pten$^{lox/lox}$ mice were crossed with FabpCrePten$^{lox/+}$ mice to generate FPTen$^{lox/lox}$, FPTen$^{lox/+}$, and FPTen$^{+/-}$ offspring that were used in the analyses as homozygous mutant, heterozygous mutant, and wild-type (WT) mice, respectively. Pten$^{lox/lox}$ mice were also occasionally used as WT controls because FPTen$^{lox/lox}$ and Pten$^{lox/lox}$ mice were indistinguishable in pilot experiments examining histology, bromodeoxyuridine (BrdUrd) incorporation, and frequency of N-butyl-N-(4-hydroxybutyl) nitrosamine (BNN)–induced bladder cancer development. The Institutional Review Board of the Akita University School of Medicine approved all animal experiments.

Preparation of bladder epithelial cells. Mouse bladders were minced with scissors and treated with 1,000 units/mL dispase (Grodoshezei, Tokyo, Japan) overnight at 4°C. The epithelium was peeled off the bladder wall and trypsinized to produce a single-cell suspension. These cells were suspended in DMEM containing 10% FCS, filtered through a Cell Strainer (Becton Dickinson, Bedford, MA) as described (25), and counted by Giemsa nuclear staining. Filtered cells were used for quantitative PCR. For Western blot analyses, single-cell suspensions of nontumorous bladder epithelial cells from FPTen$^{lox/-}$ and Pten$^{lox/lox}$ mice were cultured in DMEM containing 10% FCS for an additional 18 hours. Floating dead cells were removed and the remaining live adherent cells were subjected to Western blotting.

PCR analysis of Pten genotypes. Genomic DNA from the tails of 8-week-old mice or from bladder epithelial cells was amplified by PCR as described (23). Sense primer 5'-GTGAACTGCCCCAGATAAGG-3' (Supplementary Fig. S1A) and antisense primer 5'-CTCCCACCAATGAA-3' (Supplementary Fig. S1A) were used to detect WT and floxed Pten alleles; sense primer 5'-GGCTTAGTACCTAGTAGFC-3' (Supplementary Fig. S1Aa) and antisense primer 5'-CTCCCCACAAATGCAAACAGTC-3' (Supplementary Fig. S1Aa) were used to detect the FtenA allele; and sense primer 5'-CTAGAGATGTGATTCACATG-3' and antisense primer 5'-CGGTTATCTAATTGACC-3' were used to detect the FabpCre transgene. Amplified fragments of 428 bp (WT Pten allele), 514 bp (Pten$^{lox}$ allele), 705 bp (Pten$^{A}$ allele), and ~850 bp (FabpCre) were obtained.

Western blotting. Total lysates (20 μg) of cultured bladder epithelial cells from 8-week-old mice or bladder tumor cells were analyzed by Western blotting using antibodies directed against the following proteins (Cascade Biosciences, Winchester, MA or Cell Signaling Technology, Danvers, MA): phosphorylated Akt (Ser473), total Akt, phosphorylated ERK (Thr202/Tyr204), total ERK (all from Cell Signaling Technology); p53 (DAKO, Glostrup, Denmark); phospho-p21WAF1 and p21WAF1 (Biosciences, Winchester, MA or Cell Signaling Technology, Danvers, MA); phospho-p27KIP1 and p27KIP1 (Cell Signaling Technology, Danvers, MA); and total Rb, p21, Gli1, or actin (all from Santa Cruz Biotechnology, Santa Cruz, CA).

Flow cytometry. To estimate cell size, single cells obtained from dissected bladder epithelial layers were subjected to flow cytometry using a FACScalibur (Becton Dickinson) and individually analyzed by evaluating forward scatter (FSC). To determine cell ploidy, cells were stained with propidium iodide (PI) and subjected to flow cytometry as described previously (26).

Immunostaining. Immunohistochemical analysis for PTEN was done as described previously (27). Human prostate carcinoma cells were stained in parallel as negative controls, whereas small ureter specimens from healthy renal transplant donors were stained as positive controls. Each tissue section was stained twice using the Cell Signaling Technology anti-PTEN antibody and again with the Cascade Biosciences anti-PTEN antibody. Immunostaining patterns and intensities were scored by two independent, blinded observers.

Induced tumorigenesis. FPTen$^{lox/lox}$ (n = 35) and FPTen$^{lox/+}$ (n = 31) mice (8-10 weeks) and their WT littermates (n = 40) were fed drinking water containing freshly prepared 0.025% (v/v) BBN (TCI America, Portland, OR) as described (28). To analyze the onset of urethelial tumors, randomly selected mice were sacrificed at week 8 (14 WT, 12 FPTen$^{lox/lox}$, and 17 FPTen$^{lox/+}$), week 16 (13 WT, 9 FPTen$^{lox/lox}$, and 12 FPTen$^{lox/+}$), and week 24 (17 WT, 12 FPTen$^{lox/lox}$, and 6 FPTen$^{lox/+}$) after BBN treatment and examined histologically. Outer protrusion scores were defined as follows: 0, no protrusion; 1, mild protrusion; 2, marked protrusion; 3, marked pedicellate protrusion. Inner invasion scores were defined as follows: 0, dysplasia or early cancers without invasion or epithelial thickening; 1, early cancers with epithelial thickening but no invasion of the submucosal layer; 2, invasion of the submucosal layer; 3, invasion of the muscle layer or deeper.

Cell proliferation. Mice (10 weeks old) were given BrdUrd (3 mg/mL; Sigma, St. Louis, MO) dissolved in drinking water for 4 days and sacrificed. Anti-BrdUrd staining was done as described (29).

Human primary bladder cancers. Bladder tumor samples were obtained from 68 patients who underwent surgery for superficial and invasive bladder cancers at the Akita University Hospital in 2003 to 2005. No patient received anticancer therapy before surgery. Patients’ sex and age and tumor number, histologic grade, and stage were obtained from medical records. The 51 male and 17 female patients had a mean age of 68.5 years (range, 38-86 years). Of these 68 cases, 19 had superficial papillary TCC (pTa; see below), 12 had CIS (pT1a), 21 had focally invasive TCC (pT1v), and 16 had more advanced TCC (pT2 or more). The tumor grade 1:2:3 ratio was 1:2:2:6. Staging was done according to the 1997 tumor-node-metastasis (TNM) classification, whereas grading was based on the WHO classification (30). Definitions of pTa to pT4: pTa, no evidence of tumor; pT1, noninvasive papillary carcinoma: pT1v, CIS; pT1, tumor invasion of submucosal connective tissue; pT2, tumor invasion of muscle; pT3, tumor invasion of perivesical tissue; pT4, tumor invasion of the wall of the prostate, uterus, vagina, pelvis, or abdomen. Definitions of tumor grades 1 to 3: grade 1, well differentiated papillary tumors with limited atypia and mitoses; grade 2, intermediate between grades 1 and 3; grade 3, lesions with marked increases in number of cell layers and cell size accompanied by prominent pleomorphism and mitoses. The Institutional Review Board of the Akita University School of Medicine approved all experiments and human samples were obtained after informed consent.

Results

Generation of FPTen$^{lox/lox}$ mice. Urothelium-specific Pten-deficient mice were generated by mating FabpCre transgenic mice (24) to Pten$^{lox}$ mice (23) in which Pten exon 5, which encodes the phosphatase domain, is flanked by loxP sequences (Supplementary Fig. S1A). FPTen$^{lox/lox}$ mice were born alive and appeared healthy. PCR examination of DNA from bladder epithelial cells of 8-week-old FPTen$^{lox/lox}$ mice confirmed that efficient Cre-mediated recombination had occurred (Supplementary Fig. S1B). Quantitation of recombination was established in pilot PCR experiments using various ratios of PtenA and Pten$^{lox}$ plasmid DNAs mixed under identical PCR conditions (Supplementary Fig. S1C). The recombination frequency in bladder epithelial cells of FPTen$^{lox/lox}$ mice was >80%. Western blot analysis of the same cells confirmed a dramatic reduction of Pten protein in the mutant urothelium (Supplementary Fig. S1D).
Development of urothelial hyperplasia and spontaneous superficial papillary TCC in the absence of Pten. Histologic examination of 8-week-old \textit{FPten}\textsuperscript{fllox/}\textit{fllox} mice revealed urothelial hyperplasia. In contrast to the urothelia of WT bladder and ureter, which are composed of only three to four cell layers (basal, intermediate, and superficial), \textit{FPten}\textsuperscript{fllox/}\textit{fllox} urothelia were significantly thicker and showed five to seven cell layers (Fig. 1A). In addition, absolute numbers of bladder epithelial cells were significantly thicker and showed five to seven cell layers (Fig. 1A). Intriguingly, although polarity was normal, the size of individual \textit{FPten}\textsuperscript{fllox/}\textit{fllox} bladder epithelial cells was greater than that of \textit{FPten}\textsuperscript{+/} bladder epithelial cells as determined by flow cytometric evaluation of FSC (Fig. 1C, top). Because this enlargement of individual cells was observed in both diploid and tetraploid fractions of the total bladder epithelial cell population, the cell size enhancement was not due to polyploidy (Fig. 1C, bottom). Thus, Pten deficiency induces a thickening of the urothelial layer that is due to increases in cell number and cell size.

We next analyzed the spontaneous onset of TCC in \textit{FPten}\textsuperscript{fllox/}\textit{fllox} mice by sacrificing individuals at about every 10 weeks from 30 weeks after birth. TCC occurred with an incidence of 10% (4 of 39) in 40- to 80-week-old \textit{FPten}\textsuperscript{fllox/}\textit{fllox} mice. The minimum time to TCC formation was 40 weeks. Of these tumors, three of four were superficial pedicellate papillary cancers (Fig. 2A-F). The fourth tumor was a pedicellate papillary cancer that had invaded the muscle layer of the bladder (pT2; Fig. 2G). Two mice exhibited hydropnephrosis (Fig. 2B) due to the presence of large cancers in the bladder or renal pelvis. All TCC featured numerous mitotic cells as exemplified in Fig. 2H. No urothelial hyperplasia or TCC were observed in bladders from 50 WT and 15 \textit{FPten}\textsuperscript{fllox/+} mice.

**Increased susceptibility of \textit{FPten}\textsuperscript{fllox/}\textit{fllox} mice to BBN-induced carcinogenesis.** To examine induced urothelial carcinogenesis, \textit{FPten}\textsuperscript{+/}, \textit{FPten}\textsuperscript{fllox/+}, and \textit{FPten}\textsuperscript{fllox/fllox} mice of 8-10 weeks of age were orally given BBN, a known initiator of urothelial carcinomas. Individual mice were sacrificed at every 8 weeks until 24 weeks and examined for urothelial tumors. Surprisingly, TCC, including CIS and dysplasia (Fig. 3A), were observed in 50% and 25%, respectively, of \textit{FPten}\textsuperscript{fllox/}\textit{fllox} mice and in 33% and 11%, respectively, of \textit{FPten}\textsuperscript{fllox/+} mice as soon as 16 weeks after BBN administration (Fig. 3B). Some TCC were associated with squamous differentiation (Fig. 3A). In contrast, CIS and dysplasia were observed in only 8% and 8%, respectively, of WT mice at 16 weeks. No significant differences were observed among the genotypes in water intake, urinary excretion, food intake, or body weight (data not shown), indicating that all animals experienced identical carcinogen exposure and that BBN was no more toxic to the mutants than to WT mice.

Detailed histologic examination revealed that all BBN-induced tumors in WT mice were nonpapillary TCC (Fig. 3A, top left and C, left, a-c), malignancies that develop from CIS. Pedicellate papillary carcinomas were not observed in WT mice (Fig. 3C, left, a-c). In contrast, 50% of tumors in \textit{FPten}\textsuperscript{fllox/}\textit{fllox} mice were pedicellate papillary carcinomas with evident outer (against the luminal side) projections (Fig. 3A, bottom left, and C, left, d-f). When the outer projection and inner invasion scores were plotted (Fig. 3C, right), \textit{FPten}\textsuperscript{fllox/}\textit{fllox} mice showed a significant increase in outer projections due to the frequent onset of pedicellate papillary carcinomas. To determine whether loss of heterozygosity (LOH) of the WT Pten allele was the mechanism of tumor onset in \textit{FPten}\textsuperscript{fllox/+} mice, we used PCR to monitor the presence of \textit{Pten} exon 5. LOH was not observed in any \textit{FPten}\textsuperscript{fllox/+} tumor (Fig. 3D). Thus, BBN accelerates the onset of urothelial malignancies in Pten-deficient mice, particularly papillary carcinomas, and this acceleration is not due to LOH of the WT Pten allele.

**Increased proliferation associated with urothelial hyperplasia.** Tissue hyperplasia can arise from either an increase in cellular proliferation or a decrease in apoptosis. TUNEL staining of WT bladders revealed very few apoptotic cells, and there was no apparent further reduction in \textit{FPten}\textsuperscript{fllox/}\textit{fllox} bladders (data not shown). To determine if cell division was increased in \textit{FPten}\textsuperscript{fllox/}\textit{fllox} bladders, urothelial cell proliferation was evaluated by BrdUrd incorporation. High levels of BrdUrd incorporation were observed in the bladder epithelial cells of both 10-week-old \textit{FPten}\textsuperscript{+/} and \textit{FPten}\textsuperscript{fllox/}\textit{fllox} mice (Fig. 4A, left). However, whereas 0.67% of WT urothelial cells were BrdUrd\textsuperscript{+}, >5.67% of \textit{FPten}\textsuperscript{fllox/}\textit{fllox} urothelial cells had incorporated BrdUrd (Fig. 4A, right). Most BrdUrd\textsuperscript{+} \textit{FPten}\textsuperscript{fllox/}\textit{fllox} cells were located in the basal layer, although some intermediate and superficial layer cells were also labeled. Thus, increased proliferation is the primary mechanism by which Pten deficiency induces hyperplasia in the mouse bladder.

**Activation of Akt and ERK in Pten-deficient urothelial cells.** Pten regulates the Akt pathway via PI3P dephosphorylation (7, 15) and the Ras/ERK pathway via FAK and Shc dephosphorylation (10). Our previous demonstrations that both Akt and ERK

---

**Figure 1.** Urothelial hyperplasia in \textit{FPten}\textsuperscript{fllox/} mice. A. H&E staining of mouse bladder sections. Left, normal bladder and ureter epithelial cells from an 8-week-old WT (+/+) mouse; right, urothelial hyperplasia of the bladder and ureter from an 8-week-old \textit{FPten}\textsuperscript{fllox/} (fllox/lox) mouse. Bar, 10 μm. B, increased cell number. Total bladder epithelial cells were counted in WT and \textit{FPten}\textsuperscript{fllox/} mice of the indicated ages. Representative of three trials using four mice per group. Columns, mean; bars, SE; *, P < 0.05, statistical differences determined using Student’s t test. C, enlarged cell size. Top, single-cell suspensions of bladder epithelial cells from 32-week-old WT and \textit{FPten}\textsuperscript{fllox/} mice were subjected to flow cytometry and FSC was determined as a measure of cell size. Data are number of cells with a given FSC value and are one trial representative of four experiments. Bottom, DNA content (ploidy; N) of the cells (top) was determined by PI staining.
are constitutively activated in Pten-deficient cells showing abnormal proliferation or apoptosis (23, 29, 31) prompted us to analyze the phosphorylation of Akt and ERK in bladder epithelial cells from 8-week-old FPten<sup>+/+</sup> and FPten<sup>floxflox</sup> mice. Phosphorylation levels of both molecules (pAkt and pERK) were significantly elevated in the latter (Fig. 4B). However, the onset of bladder cancers in mice also involves p53, Rb (5), and PATCHED (28). Significantly, Pten directly affects p53 expression.
and indirectly influences p53 (13) and Rb (32) expression. We therefore analyzed whether Fpreno/flox urothelial cells showed any abnormalities in the expression of p53, p21 (a p53 target), phosphorylated Rb, or Gli1 (a PATCHED target). However, there were no obvious differences between WT and Fpreno/flox urothelial cells in the expression of any of these molecules (Fig. 4B). Thus, in bladder cancer epithelial cells, the primary mechanism driving increased cell proliferation and consequently urothelial hyperplasia seems to be the loss of Pten-mediated regulation of Akt and ERK activation. Importantly, whereas Akt activation was consistently higher in BBN-induced tumors obtained from Fpreno/+ mice compared with those from Fpreno+/- mice, ERK activation was observed in 50% of tumors from both genotypes (Fig. 4C). Thus, the accelerated onset of TCC in Fpreno/flox/flox mice is most likely due to the activation of Akt rather than ERK.

**Frequent reduction of PTEN protein expression in human bladder carcinomas.** Mutation or deletion of PTEN DNA occurs at only a low frequency in human primary bladder cancers and in bladder cancer cell lines (16–19). A single study of PTEN protein expression in bladder cancers has been reported, in which 29 bladder cancer samples were analyzed by Western blotting. Only 13.7% patients manifested a decrease in PTEN protein expression (33). However, this study did not differentiate between cancerous and normal cells or between nuclear and cytoplasmic PTEN protein expression. To determine PTEN protein expression in the cytoplasm and nucleus of tumor cells only, we did immunostaining on samples from 68 patients with primary bladder cancers. Noncancerous bladder epithelial cells within a given tissue section (Fig. 5A) and normal urothelia in the ureters of three healthy donors (Fig. 5B, a) served as positive controls. Anti-PTEN staining of bladder epithelium was variable even in healthy donor ureter samples, but nuclear PTEN expression was consistently weakest in the basal layer. The weakest staining in normal ureter epithelial cells, or in noncancerous epithelial cells within the same tissue section, was scored as +2 (normal). PTEN expression in the cytoplasm and nuclei of bladder cancer cells ranged from absent (0) to below normal (+1) to normal (+2; Fig. 5A). Levels of PTEN protein expression in the nuclei of cancer cells were lower than in the cytoplasm (Supplementary Table S1). Whereas 47% of patients showed normal PTEN staining, the remaining 53% showed below normal or absent PTEN expression in either the cytoplasm or the nucleus (Fig. 5C). Of these, 6% showed loss of PTEN protein in both the cytoplasm and the nucleus. With respect to TCC variant type, PTEN expression in either the cytoplasm or the nucleus was reduced in 42% of superficial papillary TCC (pTa) but in only 8% of CIS (pTaG; Fig. 5D, top left). There was a statistically significant correlation between reduced PTEN protein and TNM stage or tumor grade (Fig. 5D; Supplementary Table S2). Importantly, PTEN protein was reduced or absent in 94% of advanced bladder cancer patients (pT2 or greater), particularly in the nucleus (Fig. 5D). PTEN protein did not vary significantly according to sex, age, or tumor number (Supplementary Table S2).

**Discussion**

**Pten deficiency exerts a potent tumorigenic effect on urothelium in mice.** Urothelium-specific Pten deletion in mice resulted in urothelial hyperplasia due to hyperproliferation and the onset of superficial papillary urothelial cancers in 10% of the mutant animals. In humans, the reduction or loss of PTEN protein expression was observed in 42% of superficial papillary bladder cancers (pTaG) and in 94% of advanced bladder cancers (pT2 or greater). Our urothelium-specific Pten-deficient mice may furnish a useful model for human bladder cancer in which to analyze mechanisms underlying the onset of urothelial cancers and to explore drugs for the treatment of these malignancies.

Spontaneous bladder cancers in Fpreno/flox/flox mice developed late in life (>40 weeks of age) and at low frequency. Although this late onset could be due to the extremely low proliferative rate of urothelium compared with skin and intestinal epithelia (34), it could also imply that the onset of superficial papillary bladder cancers requires secondary genetic or epigenetic events in addition to Pten deficiency. Because PTEN deficiency increases susceptibility to carcinogens, and humans are continuously exposed to these agents in the environment, loss of PTEN...
function may allow a carcinogen to cause additional gene alterations. Indeed, the same carcinogens cause different bladder tumors in rats and mice. Rats develop papillary bladder tumors regardless of the carcinogen used, and these cancers become invasive only if a large dose of carcinogen is given for a prolonged period (35). In mice, the same carcinogens cause primarily urothelial dysplasia, CIS, and nonpapillary tumors that easily become invasive (36). This species difference may be related to the fact carcinogen-induced bladder tumors show a higher frequency of H-Ras mutation and a lower frequency of p53 mutation in rats than in mice (36–38). In our study, the majority of BBN-induced bladder cancers were nonpapillary in the WT but papillary in the Pten-deficient mutants. The reduced Pten present in BBN-treated mutant mice may have led to Akt and ERK hyperactivation. This hyperactivation might then accelerate the growth of a tumor initiated by a BBN-induced p53 mutation and influence CIS and nonpapillary TCC to become papillary. It should be noted that, compared with our observations, previous studies using the BBN-induced tumor model reported higher frequencies of bladder cancers at earlier times in WT mice (28, 39). These differences may be due to the relatively low dose of BBN given in our study, the more advanced age of our mice, or the variations in genetic background.

The occurrence of bladder cancers in FpTENflox/flox mice has recently been reported (40). Consistent with our findings, Yoo et al. found that bladder epithelial cells in FpTENflox/flox mice were larger than those of FpTEN-/- mice and that the frequency of spontaneous bladder cancers was significantly lower than the incidence of other cancers in these mutant mice. Yoo et al. speculated that mechanisms other than constitutive Akt activation might be important for the onset of bladder cancers because these workers did not observe the activation of either Akt or its downstream signaling molecules in FpTENflox/flox bladder epithelial cells. However, our results clearly show that Akt is strongly activated both in spontaneous bladder cancers and in BBN-induced tumors of FpTENflox/flox mice. We therefore believe that Akt activation is crucial for the onset of murine bladder cancers. Another discrepancy arises with regard to increased bladder epithelial cell proliferation, which Yoo et al.

![Figure 5](image-url)

**Figure 5.** Reduction or absence of PTEN expression in human primary bladder cancers. A and B, representative urothelial sections immunostained for PTEN protein expression. A, left, region of normal bladder epithelium (score +2); right, adjacent region showing the epithelium of a primary bladder cancer lacking PTEN expression (score 0) in both the cytoplasm and the nucleus. Bar, 10 μm (top) and 100 μm (bottom). B, a, normal PTEN protein expression in ureter epithelial cells from a healthy donor (score +2); b, PTEN expression is absent in the nucleus (score 0) but normal (score +2) in the cytoplasm. c, PTEN is reduced (score +1) in both the nucleus and the cytoplasm. d, PTEN is absent (score 0) from both the nucleus and the cytoplasm. Bar, 5 μm. C, percentages of bladder cancer patients showing reduction or loss of PTEN expression in the cytoplasm and/or nucleus. In total, PTEN expression is either reduced or lost in 53% of bladder cancer patients. D, relationship between the level of PTEN expression in the cytoplasm and/or nucleus (as defined for C) with TNM stage (left) or tumor grade (right). Note that PTEN staining in either the cytoplasm or the nucleus is decreased in 42% of pTa patients but in only 8% of pTis patients. Nuclear PTEN expression is absent in the majority of advanced bladder cancers (pT2 or greater).
observed in FPten
\textsubscript{floxflo} mice for only 1 week after birth due to the induction of p21. In contrast, we observed increased bladder epithelial cell proliferation even in 10-week-old FPten
\textsubscript{floxflo} mice. Moreover, the total number of bladder epithelial cells was much higher in our FPten
\textsubscript{floxflo} mice at 48 weeks than at 8 weeks of age, and p21 was not induced. The loss of Akt activation coupled with p21 induction in FPten
\textsubscript{floxflo} bladder epithelial cells as observed by Yoo et al. could have occurred as the epithelial cells were peeled off the bladder wall. Alternatively, the cells may have been damaged during a cell separation procedure.

**PTEN expression in human bladder cancers.** Point mutations or LOH of PTEN DNA are observed in ~6% and 5%, respectively, of human primary bladder cancers and in 13% and 14%, respectively, of bladder cancer cell lines (16–19). These low frequencies suggest that mutation of the PTEN gene itself does not play a major role in bladder carcinogenesis. However, the function of an intact gene can be lost through promoter hypermethylation, alternative splicing of pre-mRNA, or post-translational modifications. The actual frequency of PTEN abnormalities in bladder cancers thus may have been underestimated. As well, PTEN protein exists in the nucleus, although it lacks a traditional nuclear localization signal (41). Differentiated and resting cells have shown preferential nuclear localization of PTEN (27, 42), and nucleus-specific expression of PTEN can suppress cell growth (12). Moreover, activated PI3K and functional PIP3 have also been detected within the nucleus (43). Thus, a functional P13K/PTEN pathway operates in the nucleus, and the loss of nuclear PTEN function may have tumorigenic consequences.

A recent study of PTEN protein expression in human bladder cancers concluded that reduced PTEN was evident only in 14% of patients (33). However, this analysis was done by Western blotting and did not exclude the possibility that a wide variety of cell types might have been present in the samples. Furthermore, the intracellular distribution of PTEN cannot be determined by this method. In our study, we used immunostaining to evaluate PTEN expression in the cytoplasm and nucleus of individual cells. Reduced or absent PTEN in either the cytoplasm or the nucleus was found in 36 of the 68 (53%) bladder cancer samples examined. About 6% of cases showed loss of PTEN in both the cytoplasm and the nucleus, consistent with previously reported LOH percentages reported for bladder cancer patients (16–19). These values may in fact be underestimates, because the weakest staining of PTEN in control ureter or noncancerous bladder epithelial cells was scored as normal, and the most intense PTEN staining in a cancerous tissue was taken as its score.

**Proposed mechanism for the onset of urothelial cancers.** We found that 42% of primary superficial papillary bladder cancers (pT1) showed reduced PTEN, whereas 92% of CIS (pT\textsubscript{y}) samples showed normal PTEN. This result suggests that PTEN loss initiates a fraction of superficial papillary cancers (Fig. 6, **top pathway**). In mouse models, the overexpression of genes encoding growth factor–related signaling molecules, such as H-Ras, FGFR3, and epidermal growth factor (EGF) receptor (EGFR), causes urothelial hyperplasia (44–46) that can progress to superficial papillary carcinomas when additional (unknown) genes are presumably altered. In humans, activating mutations of FGFR3 have been found in >70% of superficial papillary cancers (3). Significantly, engagement of FGFR3 or EGFR stimulates Akt and ERK signaling (47). However, the gene whose alteration pushes hyperplasia into a carcinoma remains unknown. PTEN deficiency augments the activation of Akt and ERK pathways triggered by various growth factors, including FGF and EGF (48). Furthermore, urine usually contains high concentrations of growth factors, particularly EGF (49). Thus, PTEN-deficient urothelial cells may have an enhanced susceptibility to external signaling that results in hyperproliferation, which in turn increases the chance of additional genetic alterations that could tip the balance toward malignancy.

PTEN inactivation may also play a role in the progression of CIS to nonpapillary nodular invasive TCC (Fig. 6, **bottom pathway**). We found that PTEN expression was down-regulated in the cytoplasm or nucleus of many advanced nonpapillary bladder tumor cells. Most advanced nonpapillary TCC are believed to be derived from CIS (2) that were initiated by p53 or Rb mutation (5). Subsequent inactivation of PTEN may confer on these cells a growth advantage or resistance to apoptosis that leads to invasiveness or metastasis. EGFR may also be involved in this step, because EGFR overexpression occurs in 40% to 60% of bladder cancers and EGFR expression is highest in late-stage urothelial cancers (48, 50). Both PTEN and EGFR regulate the Akt and ERK pathways, and this activation, coupled with impaired p53 and Rb functions, may spur the development of advanced nonpapillary cancers.

Our finding that PTEN deficiency may contribute to the malignancy of both superficial papillary and invasive nonpapillary bladder cancers may make this regulator an attractive target for new therapeutics designed to treat these tumors.

**Acknowledgments**


Grant support: Ministry of Education, Science, Sports and Culture, Japan, Kowa Life Science Foundation, and Suzuken Memorial Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Tetsuo Noda (Tohoku University) and Drs. Junko Sasaki and Shunsuke Takasuga (Akita University) for helpful discussions and technical expertise and Dr. Jeffrey L. Gordon (Washington University School of Medicine) for providing the FabpCre transgenic mice.
References


Hyperplasia and Carcinomas in Pten-Deficient Mice and Reduced PTEN Protein in Human Bladder Cancer Patients

Hiroshi Tsuruta, Hiroyuki Kishimoto, Takehiko Sasaki, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/17/8389

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2006/09/29/66.17.8389.DC1

Cited articles
This article cites 47 articles, 20 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/17/8389.full.html#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/66/17/8389.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.