2003 survey, the rate of regular standing voiders decreased to 43.2%. There was no significant change in daytime continence status between the two surveys. More than 95% never or only occasionally suffered daytime incontinence in the two surveys. Similarly, there was no significant change in daytime pad use between the two surveys: 37 (90.2%) and 36 (87.8%) never required a pad for daytime incontinence at the 1998 and 2003 surveys, respectively. On the other hand, 15 (34.1%) and 14 (31.8%), respectively, experienced night-time incontinence, despite regular voiding during the night. The majority of the patients got up to void once or more per night, even in the 2003 survey. Although there was no significant difference, the rate of night-time pad use decreased from 47.5% to 40%.

There was no significant change in serum creatinine level between the 1998 and 2003 surveys: 0.80 ± 0.26 (standard deviation) and 0.90 ± 0.29 mg/dL, respectively.

Discussion

The present study is unique in that functional outcome was assessed at two points with an interval of 5 years. Thus longitudinal change of voiding function was evaluated for the same patients. Overall, continence status, either at daytime or at nighttime, was stable during the study period. More than 90% of the patients had never or occasionally daytime incontinence and about one-third claimed night-time incontinence, despite regular voiding during the night. On the other hand, the number of the patients who needed regular catheterization tended to increase, suggesting deterioration of voiding function with time. Interestingly, more than one-third preferred to void at sitting position. During the study period of 5 years, there was no change in renal function.

Daytime continence was excellent, with more than 90% of the patients remaining dry, or reporting only occasional spotting. This percentage is at least equal to that of a recent meta-analysis of several types of orthotopic bladder substitutions.[2,8–12] On the other hand, Hautmann[10] indicated that daytime continence rates decrease gradually 4–5 years after neobladder reconstruction. A factor may be declining external urethral sphincter function with age.[10] Thus further observation is required.

The probable most bothersome outcome of orthotopic neobladder reconstruction is nocturnal incontinence, which is feature shared by all forms of neobladders. The reported incidence of nocturnal incontinence ranged 0–67%, with an average of 28%.[9,10] In our series, about 30% of the patients experienced night-time incontinence despite regular voiding at night and more than 40% used pads during night-time. To diminish the risk of nocturnal incontinence, the majority of our patients got up to void once or more per night. It has been suggested that sleep results in an uncompensated decreased outlet resistance secondary to lack of a reflex arc that would normally signal a full bladder.[2] Increased diuresis and shift of free water into the concentrated urine may be another factors explaining night-time incontinence.[10]

It should be noted that the number of the patients who needed intermittent catheterization tended to increase with time in our series. The number of patients with routine catheterization increased from 3 (6.4%) to 9 (19.1%). At the 2003 survey, about 35% required some form of catheterization to empty the neobladder completely. Our previous study on female neobladder patients also showed deterioration of voiding function with time.[7] However, this trend was similar both in male and female patients in the present study. Using the cohort of 209 neobladder patients with median follow up of 33 months, Stein *et al.*[13] also reported that 25% required some form of intermittent catheterization. On the other hand, Studer's group reported that permanent failure to empty the bladder was not a major problem and only 3% required catheterization after 5 years.[8] There has been wide variation in the reported rate of self-catheterization, ranging from 0 to 53%,[10] but precise pathogenesis of urinary retention or elevated postvoid residual urine requiring clean intermittent catheterization remains uncertain. Interestingly, 30–40% of the patients preferred a sitting position when voiding. To our knowledge, there have been little reports referring to voiding posture in neobladder patients. Furukawa *et al.*[14] assessed the quality of life of 37 patients, including two women, with an orthotopic ileal neobladder, and found that 58% of the cases voided in a sitting position. As reported by Mikuma *et al.*,[15] neobladder patients void by abdominal straining and relaxation of the pelvic floor musculature. It is speculated that, for some patients, a sitting position helps more efficiently to increase abdominal pressure and relax the pelvic floor musculature than a regular standing position.[16]

There was no significant change in renal function measured by serum creatinine during the 5-year study period, a finding very similar to that from other studies.[10,13] On the other hand, Madersbacher *et al.*[17] recently reported long-term outcome of ileal conduit diversion. The rate of renal functional/morphological alterations increased to 50% of those surviving longer than 15 years. They emphasize the need for more long-term studies more than a decade to determine the entire morbidity spectrum. Thus vigorous long-term follow up is warranted.

We recognize the existence of methodological limitations in the evaluation of voiding function. The study is retrospective and multi-institutional. Further, the evaluation of voiding patterns can be influenced by a physician's personal interpretation as to the quality of the results. With regard to continence, in particular, one person's threshold for leakage may be entirely different from another's, given the same circumstances. Therefore, efforts to evaluate continence should focus on the individual degree of satisfaction with the level of continence, rather than the absolute amount of urinary leakage. In this regard, patient-reported quality of life assessment may be more appropriate for the evaluation of voiding function in patients with an orthotopic neobladder.

Conclusions

We assessed the functional outcome of orthotopic ileal neobladder at two points with an interval of 5 years. Continence status, either at daytime or at nighttime, was stable during the study period. The number of the patients who

needed regular catheterization tended to increase, suggesting deterioration of voiding function with time. We plan to follow those currently included in our series to the 10-year mark.

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Original Article

Management of concomitant ureteral carcinoma *in situ* at radical cystectomy

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Objective: We conducted a retrospective study to evaluate and define the management of concomitant ureteral carcinoma *in situ* (CIS) at radical cystectomy.
Methods: For 158 consecutive patients, who underwent radical cystectomy for invasive bladder cancer, ureteral CIS missed by preoperative examinations were evaluated by intraoperative analysis of frozen sections or postoperative histological analysis. The median follow-up period was 3.4 years.
Results: In total, 15 concomitant ureteral CIS were diagnosed by intraoperative ($n = 9$) or postoperative analysis ($n = 6$). Additional ureteral resection achieved no malignancies in the final ureteral margins of eight patients. During the follow-up period, five patients (3.6%) suffered from tumor recurrence in the upper urinary tract in total, as did three (20%) among the 15 patients with ureteral CIS missed by preoperative examinations. No recurrence was identified in the eight patients with no malignancy in the final ureteral margins after additional resection. Furthermore, multivariate analysis indicated that the presence of bladder CIS was a risk factor for the presence of concomitant ureteral CIS.
Conclusions: Detection of concomitant ureteral CIS by intraoperative studies, in combination with complete resection of ureteral CIS, might be beneficial for patients with risk factors such as bladder CIS.

Key words bladder tumor, concomitant ureteral carcinoma *in situ*, intraoperative frozen section.

Introduction

Radical cystectomy has been the gold standard therapy for high-grade recurrent superficial or muscle invasive bladder cancer. Concomitant upper urinary tract tumors were found at the time of cystectomy in between 12% and 18% of patients,[1–3] and the diagnosis and treatment of concomitant ureteral tumors, especially carcinoma *in situ* (CIS), has been an obstacle. Protruding tumors of the upper urinary tract are generally easily diagnosed by preoperative evaluations, including drip infusion pyelography (DIP) and computed tomography (CT), and are treated by radical cystectomy with nephroureterectomy, although it is difficult to identify ureteral CIS by preoperative evaluations. In the late 1960s, intraoperative frozen section analysis of the ureteral margin was popularized to detect concomitant ureteral tumors, because complete resection of the contaminated ureters was expected to decrease the risk of local and distant recurrence.[4] On the other hand, in the 1990s, several studies concluded that intraoperative frozen section analysis was inaccurate and brought no benefit because of the fact that it did not lessen recurrences and improve the survival of patients.[3,5] Thus the efficacy of routine intraoperative frozen section of the ureteral margin is controversial. In the present study, we analysed retrospectively the frequency and the prognosis for patients with CIS of upper urinary tract tumors missed by preoperative evaluations, to determine whether or not intraoperative frozen section is beneficial. Furthermore, the risk factors for upper urinary tract CIS were assessed statistically.

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Materials and methods

We analysed 158 consecutive patients who underwent radical cystectomy with or without nephroureterectomy for bladder cancer at Kyoto University Hospital between January 1989 and April 2003. The clinical and pathological data which was collected from the medical records included age at cystectomy, gender, histological grading according to the World Health Organization (WHO) system, clinical and pathological staging according to the 1997 TNM classification,[6,7] and previous treatment for bladder cancer, such as intravesical instillation therapy or transurethral resection (TUR). Of the 158 patients, 122 (77.2%) were men and 36 (22.8%) were women, with the median age being 68.6 years (and the range from 43 to 87).

Preoperative evaluations of the upper urinary tract generally included ultrasonography (US), DIP, CT and, if necessary, retrograde pyelography (RP) or magnetic resonance imaging (MRI). When these preoperative evaluations demonstrated upper urinary tract tumors, nephroureterectomy was performed in combination with radical cystectomy. On

the other hand, standard radical cystectomy was performed when these preoperative evaluations failed to demonstrate upper urinary tract tumors. The frozen section analysis of ureteral margins was performed by pathologists during radical cystectomy to confirm the negative ureteral margins at the urinary diversion. When ureteral tumors were identified in frozen section analysis, a short segment of the proximal ureter was additionally resected until the absence of malignancy was confirmed. After the operation, the ureteral sections used for frozen section analysis were re-examined with 10% formaldehyde-fixation and hematoxylin–eosin staining.

Updated follow-up information was obtained from patient records until the date of loss of follow up, death or the closure of the study (December 31, 2003). The median follow-up period was 3.4 years. Thirty-three (22.3%) of the cases involved loss of follow up and a further 33 (22.3%) died from the disease. All patients received follow up every 3–6 months for the first 2–3 years, and every 6 months or annually thereafter. The follow-up evaluations at each visit included physical examination (general and local), urine cytology, US and, if necessary, CT, bone scintigraphy and chest radiography. When urine cytology was positive twice or more, or radiographic images demonstrated tumors in the upper urinary tract, we clinically diagnosed the recurrence in the upper urinary tract. The recurrence in the upper urinary tract was finally confirmed by pathological examinations of nephroureterectomy or biopsies of ureters.

To assess the risk factors of the concomitant ureteral CIS, univariate and multivariate logistic regression analyses were performed for preoperative patient characteristics, including gender, multiplicity, grade, and stage of bladder tumors, intravesical CIS, urine cytology, and frequency of the recurrence. All results were considered statistically significant if the P-value was <0.05.

Results

Among the 158 patients who were treated with radical cystectomy, concomitant upper urinary tract tumors were identified in a total of 34 patients (22%) by preoperative, intraoperative and/or postoperative examinations (Table 1). Eighteen patients were diagnosed by preoperative examinations and nine were diagnosed by intraoperative frozen section analysis. Seven ureteral tumors were missed by both pre- and intraoperative examinations, but detected by postoperative pathological studies. Histopathologically, among 34 concomitant upper urinary tract tumors, 18 were ureteral CIS, in which three, nine and six, respectively, were diagnosed by preoperative, intraoperative and postoperative studies. Thus, 15 of the 16 tumors which were missed by preoperative studies were ureteral CIS (Table 1).

As for the treatment for concomitant upper urinary tract tumors, nephroureterectomy was performed in combination with radical cystectomy in all 18 patients who were diagnosed by preoperative examinations, including the three ureteral CIS (Table 1). Among the nine patients with ureteral CIS identified by intraoperative studies, additional resection of proximal ureters achieved negative ureteral margins in eight patients but not in one. Seven patients who had tumors detected by postoperative examinations received no treatment and were subject to close follow up.

During the follow-up period, cancer developed in the upper urinary tracts of five patients, and the mean interval to recurrence was 22.2 months (ranging from 4 to 47) (Tables 1, 2). Of 124 patients without concomitant upper urinary tract tumors, two (2%) suffered from development of upper urinary tract tumors (pTis and pT2). On the other hand, of the 15 patients with concomitant ureteral CIS missed by preoperative examinations, three (20%) suffered from development of upper urinary tract tumors (Table 1). Two of the three were in a group whose concomitant ureteral tumors were diagnosed by postoperative examinations. Another one was a patient with positive final ureteral margins despite additional resection of the proximal ureter. No tumor recurrence in the upper urinary tract was demonstrated in eight patients who achieved finally negative ureteral margins after additional resection of the proximal ureters. The median follow-up period of these eight patients was 3.5 years.

Of three patients with CIS recurrence in the upper urinary tracts, two patients were alive after treatment with bacillus Calmette-Guérin instillation therapy, although one patient died of the disease after 50 months despite of treatment with nephroureterectomy (Table 2). The pathological stage of primary bladder cancer in this patient was pTis. Both of the patients with a pT2 tumor recurrence were

Table 1 Management of concomitant upper urinary tract tumors

Concomitant UUT tumors	Diagnostic procedure	No. patients	Stage pTis	Stage pTa≤	Primary treatment	Recurrence in UUT
Negative		124 (78%)	–	–	Follow up	2
Positive		34 (22%)	18	16		3
	Preoperative	18 (11%)	3	15	Concomitant nephroureterectomy	0
	Intraoperative	9 (6%)	9	0	Intraoperative additional ureteral resection	1†
	Postoperative	7 (5%)	6	1	Follow up	2‡

†This later recurrence developed in the only one patient with positive final margin. ‡These two patients with later recurrences had concomitant ureteral carcinoma *in situ*. UUT, upper urinary tract.

Table 2 Patients with tumor recurrence in upper urinary tract

Case number	Concomitant UUT tumors	Diagnostic procedure	Recurrence in UUT			
			Time to recurrence	Stage	Treatment	Prognosis
1	Positive	Intraoperative	47M	pTis	Nephroureterctomy	50M (DOD)
2	Positive	Postoperative	6M	pTis	BCG instillation	19M (NED)
3	Positive	Postoperative	4M	pT2	Nephroureterctomy	63M (NED)
4	Negative	–	38M	pTis	BCG instillation	49M (NED)
5	Negative	–	16M	pT2	Nephroureterctomy	32M (DOD)

BCG, bacillus Calmette-Guérin; DOD, death of disease; NED, no evidence of disease; UUT, upper urinary tract.

Table 3 Risk factors of concomitant ureteral CIS

	Total (%)	Ureteral CIS n (%)	Univariate		Multivariate	
			P-value	Odds ratio (95% CI)	*P*-value	Odds ratio (95% CI)
Gander						
Male	122 (77)	12 (9.8)				
Female	36 (23)	6 (16.7)	0.26	1.83 (0.64–5.29)		
Grade						
G1–2	44 (34)	6 (13.6)				
G3	86 (66)	11 (12.8)	0.89	0.93 (0.32–2.70)		
Stage						
≤pT2	70 (47)	13 (18.6)				
≥pT3	78 (53)	4 (5.1)	0.02	0.24 (0.07–0.77)	0.14	0.39 (0.11–1.35)
Multiplicity						
Solitary	86 (57)	5 (5.8)				
Multiple	65 (43)	12 (18.5)	0.02	3.67 (1.22–11.01)	0.28	1.92 (0.59–6.32)
Cytology						
Negative	23 (18)	1 (4.3)				
Positive	106 (82)	16 (15.1)	0.19	3.91 (0.49–31.25)		
Intravesical CIS						
Absent	94 (80)	9 (9.6)				
Present	24 (20)	8 (33.3)	0.01	4.72 (1.58–14.08)	0.04	3.33 (1.05–10.53)
Past history						
Primary	71 (55)	6 (8.5)				
Recurrent	58 (45)	8 (13.8)	0.34	1.73 (0.56–5.32)		

CIS, carcinoma *in situ*.

treated with nephroureterectomy but one died of the disease after 32 months.

To evaluate the risk factors for concomitant ureteral CIS, preoperative parameters were assessed. Univariate logistic regression analysis demonstrated that a lower pathological stage of bladder cancer (pT2; $P = 0.02$, odds ratio [OR] = 0.24, 95% confidence interval [CI] = 0.07–0.77), multiplicity of bladder cancer at radical cystectomy ($P = 0.02$, OR = 3.67, 95% CI = 1.22–11.01) and the presence of concomitant CIS in the bladder ($P = 0.01$, OR = 4.72, 95% CI = 1.58–14.08) were significantly associated with the presence of ureteral CIS, whereas gender (male vs female), urine cytology (negative vs positive), past history of superficial bladder cancer (primary vs recurrent) and tumor grade (grade 1–2 vs grade 3) were not related (Table 3). Multivariate analysis demonstrated that the presence of concomitant CIS in the bladder ($P = 0.04$, OR = 3.33, 95% CI = 1.05–10.53) was only associated with ureteral CIS.

Discussion

In the present study, we analysed retrospectively the frequencies of concomitant upper urinary tract tumors at radical cystectomy for bladder tumors which were performed within these 15 years. It is an obstacle at radical cystectomy to deal with concomitant upper urinary tract tumors, especially ureteral CIS. The frequencies of concomitant tumors and CIS in upper urinary tracts have been reported to be 12–18%[8,9] and 2–8%,[1–3] respectively. In our series, the frequencies of the concomitant ureteral tumors and ureteral CIS were 21.5% and 11.4%, respectively, which were consistent with the results of previous reports.[1–3,8,9] Generally, imaging techniques have advanced during the past 10 years and have increased sensitivity and specificity for staging and invasion-sites of bladder tumors. In our institute, upper urinary tract tumors were assessed routinely by DIP and CT and, if necessary, several further studies, including MRI, RP and washing cytology for

upper urinary tracts, were performed. These preoperative studies successfully identified almost all protruding upper urinary tract tumors but not more than 80% of ureteral CIS. Thus the preoperative diagnosis for CIS in the upper urinary tract was still a major problem at radical cystectomy, despite recent advances in imaging techniques.

To identify ureteral CIS at radical cystectomy, intraoperative frozen section analysis of the ureteral margin was popularized in the late 1960s, although several studies in the 1990s pointed out the inaccuracy of intraoperative frozen section analysis. Large population studies of more than 100 patients demonstrated that intraoperative frozen section analysis identified ureteral CIS in ureteral margin of approximately 8% of patients. These reports demonstrated that false positive and false negative rates were 2% and 6%, respectively.[3] Our findings (false positive rate: 0% (0/9), and false negative rate: 5% (7/131), respectively) were consistent with those data, indicating that frozen section analysis possessed limited ability to detect ureteral CIS. On the other hand, one of the features of the intraoperative frozen section analysis is to identify ureteral CIS in ureteral margin and to make it possible to resect the proximal ureteral segment in such cases. Hypothetically, an additional resection of ureteral segments facilitates complete removal of cancer and reduces tumor recurrence in the upper urinary tract, but its hypothetical benefit remains controversial.[3,5] The frequencies of tumor development in the upper urinary tract after cystectomy were 2–4% in past reports.[10,11] Mark *et al.* described that only one of 101 patients with carcinoma *in situ* of the ureteral margin had upper urinary tract recurrence.[3] Linker *et al.* suggested that ureteral carcinoma *in situ* encountered at cystectomy probably had little overall effect on the clinical outcome.[5] In our series, the overall rate of tumor development in upper urinary tracts was 3.1% (5/158 patients), which is consistent with the rates of past reports. Interestingly, upper urinary tract tumors arose in three patients (20%) among 15 with concomitant ureteral CIS and both of the patients with positive margins diagnosed by frozen section had experienced clinical development of ureteral cancer. However, no tumor development was found in all eight patients who showed no malignancy in final ureteral margins. These results demonstrated that sequential resection of ureteral margin might reduce the recurrence in upper urinary tracts, even if a positive ureteral margin was diagnosed by frozen section.

Another problem of intraoperative frozen section analysis is the low incidence of ureteral CIS, which is approximately 8%. Schoenberg *et al.* reported that CIS in ureters were most frequently observed in patients with high stage and high grade bladder cancer, and recommended intraoperative frozen section analysis at radical cystectomy only for high risk patients.[3] Jonson and Cooper reported that CIS of the ureteral margin was most frequently observed in patients with diffuse intravesical CIS, positive ductal involvement, and high grade and high stage cancer.[1,9] In our study, eight (47%) of 17 patients with ureteral CIS had concomitant intravesical CIS, and unanticipated ureteral CIS at radical cystectomy was statistically associated with patients with multiple tumors, lower stage cancer and intravesical CIS. Our results, in combination with the previous data, supported the recommendation that intraoperative frozen section analysis should be performed, especially in high risk patients with intravesical CIS.

Several mechanisms have been proposed for the development of tumors in the lower ureteral segments in relation to bladder tumors: dissemination of upper urinary tract cancer cells to the bladder, reflux of bladder cancer cells to the upper urinary tract, field cancerization of the whole urinary tract epithelium, and the consecutive spreading of CIS of the bladder to the ureter.[8,12] Koss *et al.* meticulously examined surgical specimens of bladders and ureters in radical cystectomy and found that concomitant carcinoma *in situ* of the ureter was consecutive from the bladder.[12] As well, Culp *et al.* reported that unidentified CIS could result in a recurrence in the remaining ureter.[8] Our findings that additional resection of proximal ureteral segments decreased tumor development in the upper urinary tract and that the incidence of ureteral CIS was related to intravesical CIS might support the hypothesis that CIS in lower ureteral segments is spread intramucously from intravesical tumors (especially CIS).

Conclusions

The existence of concomitant ureteral CIS at radical cystectomy for invasive bladder cancer can be predicted by several preoperative variables, including intravesical CIS. For high-risk patients, intraoperative frozen-section analysis of ureteral stump is important for predicting later clinical development of ureteral cancer.

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

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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 [*FabpCrePten*$^{flox/flox}$ (*FPten*$^{flox/flox}$) mice]. Histologic examination revealed that all *FPten*$^{flox/flox}$ mice exhibited urothelial hyperplasia in which component cells showed enlarged nuclei and increased cell size. With time, 10% of *FPten*$^{flox/flox}$ mice spontaneously developed pedicellate papillary transitional cell carcinomas (TCC). This type of tumor also arose in *FPten*$^{flox/flox}$ mice treated with the chemical carcinogen *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. *FPten*$^{flox/flox}$ 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 advanced 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

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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*$^{flox/flox}$ (*FPten*$^{flox/flox}$) 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*$^{flox/flox}$ mice. *Pten*$^{flox/flox}$ mice (129Ola × 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*$^{flox/flox}$ mice were crossed with *FabpCrePten*$^{flox/+}$ mice to generate *FPten*$^{flox/flox}$, *FPten*$^{flox/+}$, and *FPten*$^{+/+}$ offspring that were used in the analyses as homozygous mutant, heterozygous mutant, and wild-type (WT) mice, respectively. *Pten*$^{flox/flox}$ mice were also occasionally used as WT controls because *FPten*$^{+/+}$ and *Pten*$^{flox/flox}$ mice were indistinguishable in pilot experiments examining histology, bromodeoxyuridine (BrdUrd) incorporation, and frequency of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN)–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 (Godoshusei, 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*$^{+/+}$ and *Pten*$^{flox/flox}$ 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′-GTGAAAGTGCCCCAACATAAGG-3′ (Supplementary Fig. S1Ab) and antisense primer 5′-CTCCCACCAATGAACAAACAGT-3′ (Supplementary Fig. S1Ac) were used to detect the WT and floxed *Pten* alleles; sense primer 5′-GGCCTAGGACTCACTAGATAGC-3′ (Supplementary Fig. S1Aa) and antisense primer 5′-CTCCCACCAATGAACAAACAGT-3′ (Supplementary Fig. S1Ac) were used to detect the *PtenΔ* allele; and sense primer 5′-CTAGAGATGTGATTCACATG-3′ and antisense primer 5′-CCGGTTATTCAACTTGCACC-3′ were used to detect the *FabpCre* transgene. Amplified fragments of 428 bp (WT *Pten* allele), 514 bp (*Pten*^{flox} allele), 705 bp (*PtenΔ* 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: PTEN (Cascade Biosciences, Winchester, MA or Cell Signaling Technology, Danvers, MA); phosphorylated Akt (Ser^{473}), total Akt, phosphorylated ERK (Thr^{202}/Tyr^{204}), and total ERK (all from Cell Signaling Technology); p53 (DAKO, Glostrup, Denmark); phosphorylated Rb (Ser^{249}/Thr^{252}), 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*$^{flox/flox}$ (n = 35) and *FPten*$^{flox/+}$ (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 urothelial tumors, randomly selected mice were sacrificed at week 8 (14 WT, 12 *FPten*$^{flox/+}$, and 17 *FPten*$^{flox/flox}$), week 16 (13 WT, 9 *FPten*$^{flox/+}$, and 12 *FPten*$^{flox/flox}$), and week 24 (17 WT, 12 *FPten*$^{flox/+}$, and 6 *FPten*$^{flox/flox}$) 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 (pT_a; see below), 12 had CIS (pT_{is}), 21 had focally invasive TCC (pT_1), and 16 had more advanced TCC (pT_2 or more). The tumor grade 1:2:3 ratio was 1:2.1:2.6. Staging was done according to the 1997 tumor-node-metastasis (TNM) classification, whereas grading was based on the WHO classification (30). Definitions of pT_0 to pT_4: pT_0, no evidence of tumor; pT_a, noninvasive papillary carcinoma; pT_{is}, CIS; pT_1, tumor invasion of subepithelial connective tissue; pT_2, tumor invasion of muscle; pT_3, tumor invasion of perivesical tissue; pT_4, 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*$^{flox/flox}$ mice. Urothelium-specific Pten-deficient mice were generated by mating *FabpCre* transgenic mice (24) to *Pten*^{flox} mice (23) in which *Pten* exon 5, which encodes the phosphatase domain, is flanked by *loxP* sequences (Supplementary Fig. S1A). *FPten*$^{flox/flox}$ mice were born alive and appeared healthy. PCR examination of DNA from bladder epithelial cells of 8-week-old *FPten*$^{flox/flox}$ 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 *PtenΔ* and *Pten*^{flox} plasmid DNAs mixed under identical PCR conditions (Supplementary Fig. S1C). The recombination frequency in bladder epithelial cells of *FPten*$^{flox/flox}$ 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 *FPten*$^{flox/flox}$ 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), *FPten*$^{flox/flox}$ urothelia were significantly thicker and showed five to seven cell layers (Fig. 1*A*). In addition, absolute numbers of bladder epithelial cells were increased 1.6-fold over WT levels at 8 weeks and 2.6-fold at 48 weeks (Fig. 1*B*). Intriguingly, although polarity was normal, the size of individual *FPten*$^{flox/flox}$ bladder epithelial cells was greater than that of *FPten*$^{+/+}$ bladder epithelial cells as determined by flow cytometric evaluation of FSC (Fig. 1*C*, *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. 1*C*, *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 *FPten*$^{flox/flox}$ 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 *FPten*$^{flox/flox}$ mice. The minimum time to TCC formation was 40 weeks. Of these tumors, three of four were superficial pedicellate papillary cancers (Fig. 2*A-F*). The fourth tumor was a pedicellate papillary cancer that had invaded the muscle layer of the bladder (pT_2; Fig. 2*G*). Two mice exhibited hydronephrosis (Fig. 2*B*) due to the presence of large cancers in the bladder or renal pelvis. All TCC featured numerous mitotic cells as exemplified in Fig. 2*H*. No urothelial hyperplasia or TCC were observed in bladders from 50 WT and 15 *FPten*$^{flox/+}$ mice.

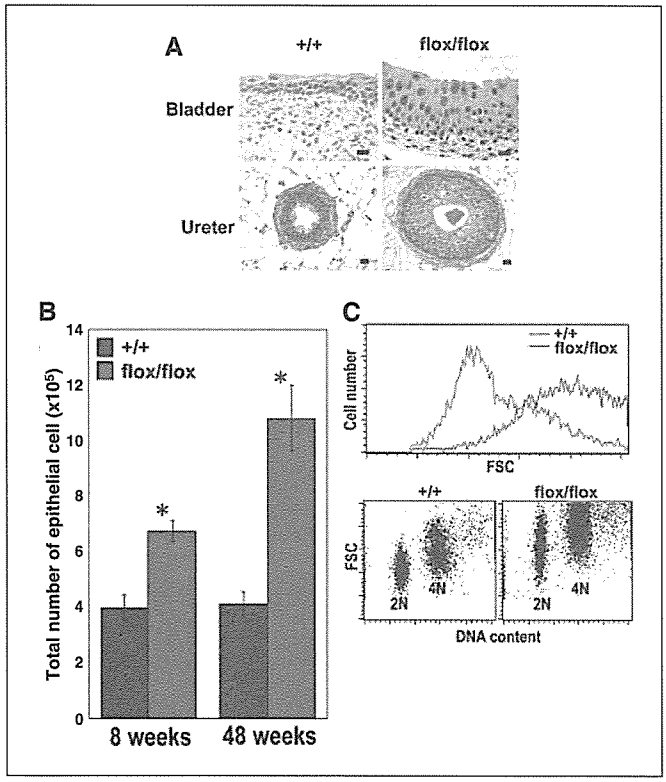

Figure 1. Urothelial hyperplasia in *FPten*$^{flox/flox}$ mice. *A*, H&E staining of mouse bladder sections. *Left*, normal bladder and ureter epithelial cells from an 8-week-old WT (+/+) mouse; *right*, urothelial hyperplasia of the bladder and ureter from an 8-week-old *FPten*$^{flox/flox}$ (*flox/flox*) mouse. Bar, 10 μm. *B*, increased cell number. Total bladder epithelial cells were counted in WT and *FPten*$^{flox/flox}$ 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 *FPten*$^{flox/flox}$ 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.

Increased susceptibility of *FPten*$^{flox/flox}$ mice to BBN-induced carcinogenesis. To examine induced urothelial carcinogenesis, *FPten*$^{+/+}$, *FPten*$^{flox/+}$, and *FPten*$^{flox/flox}$ 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. 3*A*), were observed in 50% and 25%, respectively, of *FPten*$^{flox/flox}$ mice and in 33% and 11%, respectively, of *FPten*$^{flox/+}$ mice as soon as 16 weeks after BBN administration (Fig. 3*B*). Some TCC were associated with squamous differentiation (Fig. 3*A*). 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. 3*A*, *top left* and *C*, *left*, *a-c*), malignancies that develop from CIS. Pedicellate papillary carcinomas were not observed in WT mice (Fig. 3*C*, *left*, *a-c*). In contrast, 50% of tumors in *FPten*$^{flox/flox}$ mice were pedicellate papillary carcinomas with evident outer (against the luminal side) projections (Fig. 3*A*, *bottom left*, and *C*, *left*, *d-f*). When the outer projection and inner invasion scores were plotted (Fig. 3*C*, *right*), *FPten*$^{flox/flox}$ 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 *FPten*$^{flox/+}$ mice, we used PCR to monitor the presence of *Pten* exon 5. LOH was not observed in any *FPten*$^{flox/+}$ tumor (Fig. 3*D*). 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 *FPten*$^{flox/flox}$ bladders (data not shown). To determine if cell division was increased in *FPten*$^{flox/flox}$ 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 *FPten*$^{+/+}$ and *FPten*$^{flox/flox}$ mice (Fig. 4*A*, *left*). However, whereas 0.67% of WT urothelial cells were BrdUrd$^+$, >5.67% of *FPten*$^{flox/flox}$ urothelial cells had incorporated BrdUrd (Fig. 4*A*, *right*). Most BrdUrd$^+$ *FPten*$^{flox/flox}$ 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 PIP3 dephosphorylation (7, 15) and the Ras/ERK pathway via FAK and Shc dephosphorylation (10). Our previous demonstrations that both Akt and ERK

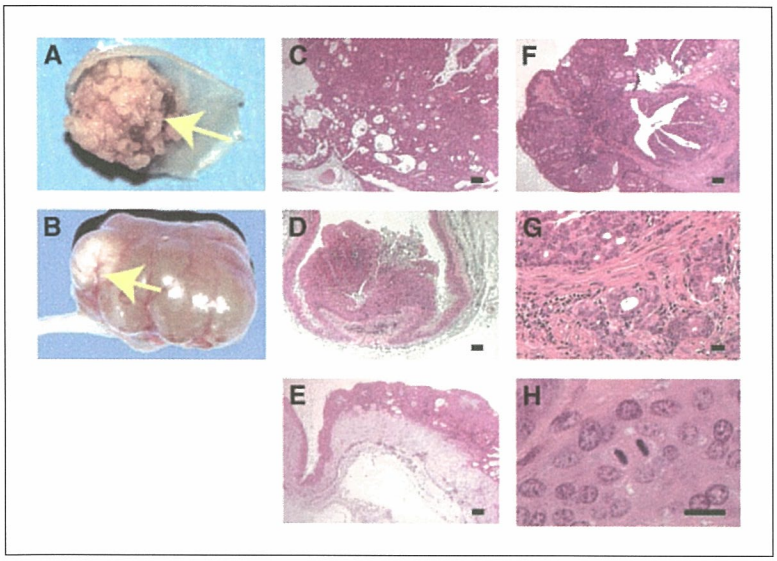

Figure 2. Spontaneous superficial papillary carcinomas in *FPten*$^{flox/flox}$ mice. Gross and histologic analyses of urothelial cancers in *FPten*$^{flox/flox}$ mice. *A* and *B,* gross appearance of a pedicellate papillary bladder carcinoma (*A; arrow*) and a pelvic carcinoma (*B; arrow*) exhibiting hydronephrosis. The renal parenchyma in (*B*) was severely atrophic due to the hydronephrosis. *C* to *E,* histology of pedicellate superficial papillary carcinomas (pT_a) observed at week 76 (*C*; same mouse as in *A*), week 48 (*D*), and week 47 (*E*) in mutant mice. *F* and *G,* histologic analysis of a section of the papillary carcinoma in (*B*) showing tumor cell infiltration into the muscle layer (pT_2) at 47 weeks. Magnification, ×5 (*F*) and ×200 (*G*). *H,* frequent mitotic figures observed in the pedicellate papillary bladder carcinoma in (*A*). Bars, 100 μm (*C-F*) and 10 μm (*G* and *H*).

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*$^{+/+}$ and *FPten*$^{flox/flox}$ mice. Phosphorylation levels of both molecules (pAkt and pERK) were significantly elevated in the latter (Fig. 4*B*). However, the onset of bladder cancers in mice also involves p53, Rb (5), and PATCHED (28). Significantly, Pten directly affects p53 expression

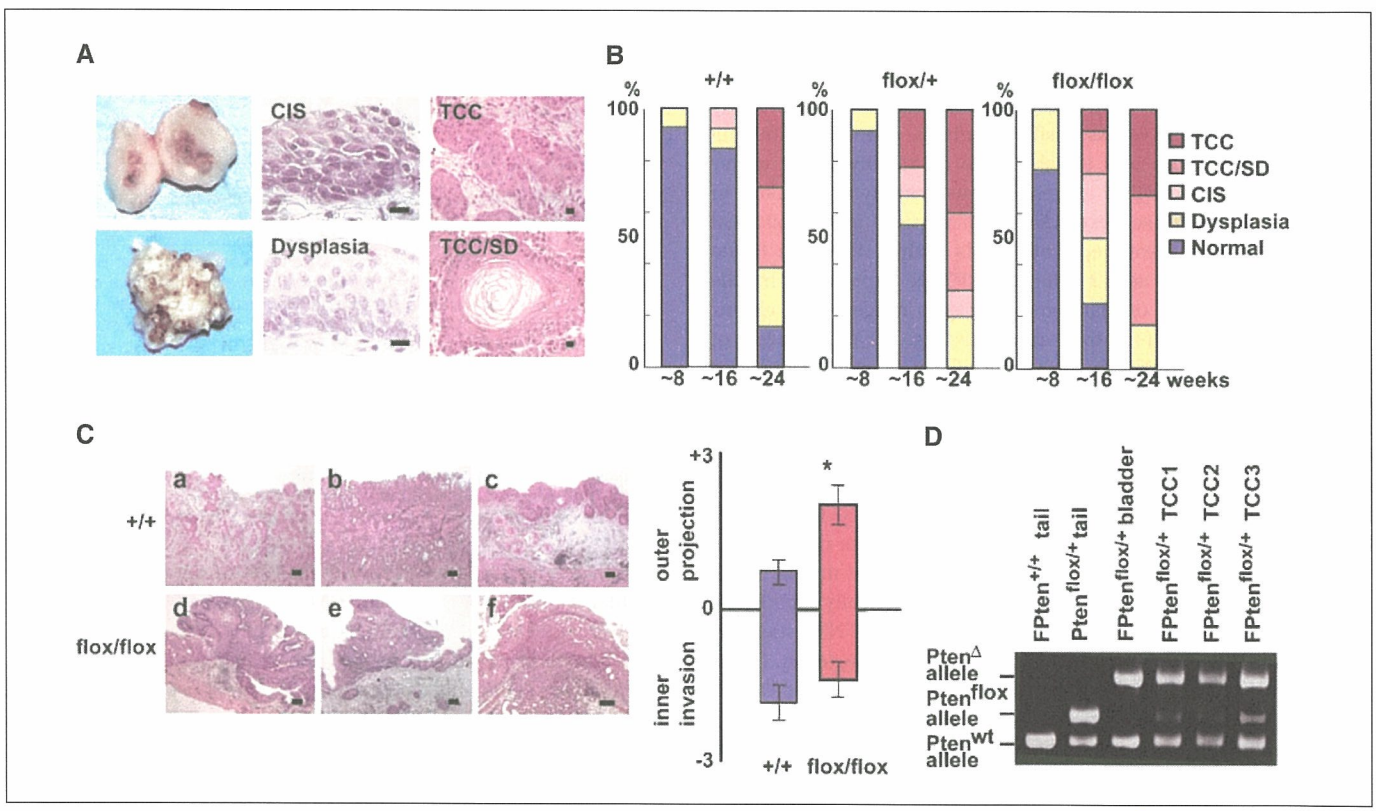

Figure 3. Acceleration of BBN-induced urothelial tumorigenesis. *A,* representative tumors observed following BBN administration. *Left,* gross appearance of tumors observed in WT (*top*) and *FPten*$^{flox/flox}$ (*bottom*) mice. *Middle* and *right,* dysplasia, CIS, TCC, and TCC with squamous cell differentiation (*TCC/SD*) from *FPten*$^{flox/flox}$ mice. Bar, 10 μm. *B,* increased susceptibility of mutant mice to BBN. Histograms show the proportions of dysplastic and neoplastic changes in WT, *FPten*$^{flox/+}$ (*flox/+*), and *FPten*$^{flox/flox}$ mice at the indicated times after BBN treatment. *C,* frequent onset of pedicellate superficial papillary carcinomas in *FPten*$^{flox/flox}$ mice. *Left,* histology of bladder tumors from WT and *FPten*$^{flox/flox}$ mice at 24 weeks after BBN administration. Compared with WT mice (*top row*), pedicellate superficial papillary carcinomas occurred at increased frequency in *FPten*$^{flox/flox}$ mice (*bottom row*). Bar, 100 μm. *Right,* plotting of the outer protrusion and inner invasion scores of the tumors from WT and *FPten*$^{flox/flox}$ mice in (*C, left*). *Columns,* mean score of eight carcinomas per group; *bars,* SE. *, $P < 0.05$, statistical differences determined using Student's *t* test. *D,* lack of LOH in *FPten*$^{flox/+}$ mice. Quantitative genomic PCR was used to analyze the presence of the WT Pten allele in the indicated types of tumors from *FPten*$^{flox/flox}$ mice. *FPten*$^{flox/+}$ tail and *FPten*$^{flox/flox}$ tail DNA were analyzed as controls. PCR conditions were identical to those in Fig. 1*B*.

(13) and indirectly influences p53 (13) and Rb (32) expression. We therefore analyzed whether *FPten*$^{flox/flox}$ 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 *FPten*$^{flox/flox}$ urothelial cells in the expression of any of these molecules (Fig. 4*B*). 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 *FPten*$^{flox/flox}$ mice compared with those from *FPten*$^{+/+}$ mice, ERK activation was observed in 50% of tumors from both genotypes (Fig. 4*C*). Thus, the accelerated onset of TCC in *FPten*$^{flox/flox}$ 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. 5*A*) and normal urothelia in the ureters of three healthy donors (Fig. 5*B, 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. 5*B, b-d*). 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. 5*C*). 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 (pT_a) but in only 8% of CIS (pT_{is}; Fig. 5*D, top left*). There was a statistically significant correlation between reduced PTEN protein and TNM stage or tumor grade (Fig. 5*D*; Supplementary Table S2). Importantly, PTEN protein was reduced or absent in 94% of advanced bladder cancer patients (pT_2 or greater), particularly in the nucleus (Fig. 5*D*). PTEN protein did not vary significantly according to sex, age, or tumor number (Supplementary Table S2).

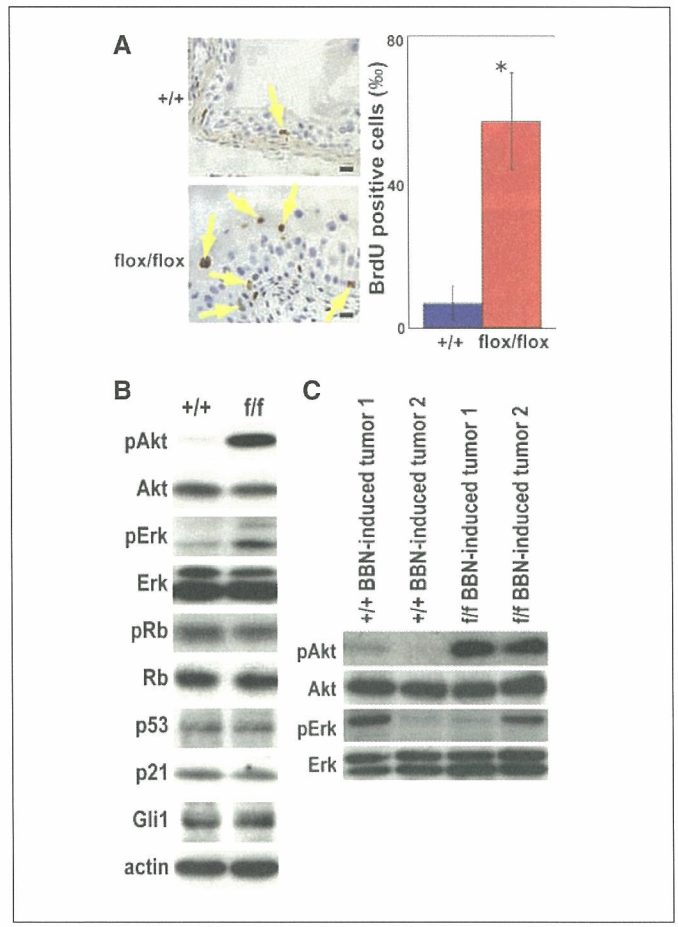

Figure 4. Increased bladder epithelial cell proliferation and enhanced phosphorylation of Akt and ERK in *FPten*$^{flox/flox}$ mice. *A,* increased bladder epithelial cell proliferation. *Left,* BrdUrd$^+$ bladder epithelial cells (*arrows*) from 10-week-old WT and *FPten*$^{flox/flox}$ mice were counted 4 days after BrdUrd administration. Bar, 10 μm. *Right,* percentage of BrdUrd$^+$ epithelial cells per 5×10^2 bladder epithelial cells per mouse. Representative of three trials using four mice per group. *Columns,* mean; *bars,* SE. *B,* increased phosphorylation of Akt and ERK. The phosphorylated forms of Akt, ERK1/2, and Rb and the expression of p53, p21, and Gli1 were detected by immunoblotting of lysates of bladder epithelial cells from 8-week-old WT and *FPten*$^{flox/flox}$ (*f/f*) mice. Total Akt, total ERK, and actin were evaluated as controls. *C,* Akt and ERK activation in BBN-induced tumors. WT and *FPten*$^{flox/flox}$ mice were treated with BBN as described in Materials and Methods and the phosphorylation of Akt and ERK was evaluated in tumors by immunoblot. Akt activation was consistently higher in tumors from *FPten*$^{flox/flox}$ mice than in those from WT mice, but there was no difference between the genotypes in ERK activation in tumors.

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 (pT_a) and in 94% of advanced bladder cancers (pT_2 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 *FPten*$^{flox/flox}$ 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 *FPten*$^{flox/flox}$ mice has recently been reported (40). Consistent with our findings, Yoo et al. found that bladder epithelial cells in *FPten*$^{flox/flox}$ 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 *FPten*$^{flox/flox}$ bladder epithelial cells. However, our results clearly show that Akt is strongly activated both in spontaneous bladder cancers and in BBN-induced tumors of *FPten*$^{flox/flox}$ 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.

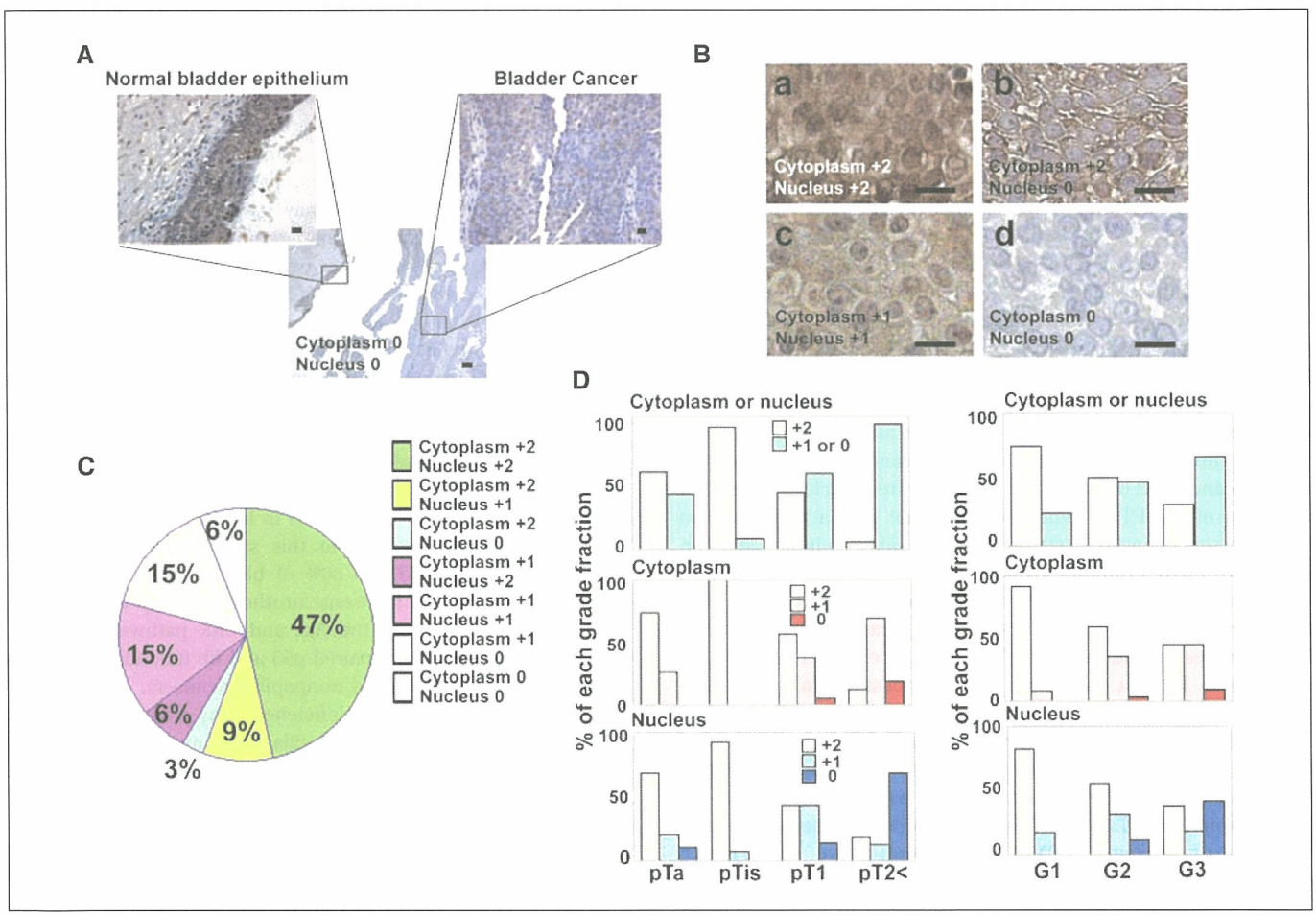

Figure 5. Reduction or absence of PTEN expression in human primary bladder cancers. *A* and *B,* representative urothelial sections immunostained for PTEN protein expression. *A, left,* region of normal bladder epithelium (score +2); *right,* adjacent region showing the epithelium of a primary bladder 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 pT_a patients but in only 8% of pT_{is} patients. Nuclear PTEN expression is absent in the majority of advanced bladder cancers (pT_2 or greater).

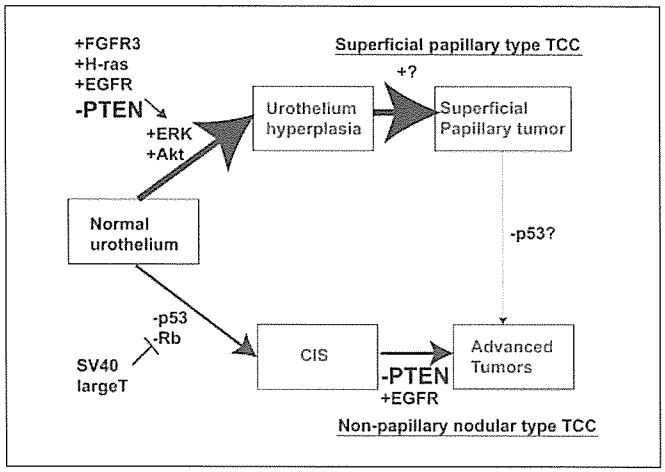

Figure 6. Model of bladder cancer progression. *Top pathway,* overexpression of FGFR3, EGFR, or H-Ras, coupled with loss of PTEN, promotes cell proliferation by activating the Akt and ERK pathways. Urothelial hyperplasia is induced that can progress to superficial papillary TCC following additional (unknown) genetic alterations. If p53 function is subsequently lost, these malignancies may become advanced tumors. *Bottom pathway,* overexpression of SV40, which inactivates p53 and Rb, induces CIS development. Under the influence of EGFR overexpression and/or PTEN loss, CIS cells may acquire a growth advantage and progress to invasive and metastatic nonpapillary nodular type TCC.

observed in *FPten*$^{flox/flox}$ 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*$^{flox/flox}$ mice. Moreover, the total number of bladder epithelial cells was much higher in our *FPten*$^{flox/flox}$ 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*$^{flox/flox}$ 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 PI3K/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 (pT_a) showed reduced PTEN, whereas 92% of CIS (pT_{is}) 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.

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Surgical treatment for urethral recurrence after ileal neobladder reconstruction in patients with bladder cancer

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OBJECTIVE

To report a retrospective study evaluating the management of superficial urethral recurrence after ileal neobladder construction in patients with bladder cancer.

PATIENTS AND METHODS

In 77 consecutive patients with ileal neobladder after radical cystectomy for invasive bladder cancer, urethral recurrence was evaluated and transurethral resection (TUR) used as an initial treatment for superficial urethral recurrence. Urethrectomy with urinary re-diversion was performed when further recurrence developed.

RESULTS

Four patients (5%) presented with a superficial urethral recurrence and all four were treated by TUR as initial therapy. One patient has had no evidence of recurrence after initial TUR, although the other three patients were later treated with salvage urethrectomy due to repeated urethral recurrence. As a result, the stage of urethral recurrence advanced from pTa to pT1–pT2 in two of the three patients. For urinary re-diversion, one patient had a conversion from a Studer pouch to an ileal conduit, using the afferent limb, and the other two were converted from a Hautmann pouch to a continent reservoir using the Appe-Mainz procedure. There was no evidence of metastasis or local recurrence in any of the four patients.

CONCLUSION

Urethral preservation at initial therapy for superficial recurrence might be reasonable, and sequential urethrectomy after attempted urethral preservation might be strategically feasible. Urinary re-diversion from a neobladder to a catheterizable continent reservoir using the appendix would be a good choice and maintains the quality of life.

KEYWORDS

bladder cancer, ileal neobladder, urethral recurrence, urethrectomy, urinary diversion

INTRODUCTION

In contemporary series, tumour development in the urethra (urethral recurrence) has been reported in 2–6% of patients with an ileal neobladder reconstruction after radical cystectomy for bladder cancer [1–3]. The prognosis of urethral recurrence is reported to be generally poor, with a 35.2% 5-year actuarial overall survival, even if urethrectomy and chemotherapy is used [4]. However, when tumours in the urethra are superficial, transurethral resection (TUR) and/or intraurethral BCG instillation have recently been used to preserve the urethra and thus maintain a high quality of life. There are few reports of the outcome of conservative therapy for urethral recurrence, as this entity is rare [4]. When performing urethrectomy, urinary diversion becomes an obstacle, due to adhesions caused by previous surgery. Herein, we report our experience with surgical treatment in four patients with a superficial urethral recurrence after ileal neobladder reconstruction.

PATIENTS AND METHODS

Seventy-seven patients had a radical cystectomy with an ileal neobladder (23 a Studer pouch and 54 a Hautmann pouch) for bladder cancer, at Kansai Medical University and Kyoto University, between January 1993 and June 2004. Generally, exclusion criteria for ileal neobladder reconstruction in our institutions have been age >80 years, poor performance status, tumour invasion into the prostatic urethra or positive intraoperative frozen sections of the urethral margin.

Updated follow-up information was obtained from patient records until the date of loss to follow-up, death or closure of the study (31 June 2005). The median follow-up was 60 months. Five patients (6%) were lost to follow-up and 11 (14%) died from the disease. All patients were followed up every 3–6 months for the first 2–3 years, and every 6 months or annually thereafter. Generally, the follow-up evaluation at each visit included a physical examination, urinary cytology, ultrasonography and, if necessary, CT, bone scintigraphy and chest radiography. When patients had positive urinary cytology, macrohaematuria or voiding symptoms, we used cysto-urethroscopy to exclude urethral recurrence.

When tumour developed in the urethra, transurethral biopsies were taken to decide the pathological staging, after radiological examinations including CT and/or MRI. When the recurrent urethral tumours were superficial, they were treated by TUR or resection with a YAG laser. After the first recurrence the patients were followed up by cytology and urethroscopy. When recurrent tumours were invasive or repeatedly recurrent, we recommended urethrectomy with urinary re-diversion.

In this series, after urethrectomy two types of urinary re-diversion were used; one was conversion from the Hautmann pouch [5] to a continent reservoir, using the appendix as a continence mechanism (Fig. 1a,b), and the

FIG. 1. Urinary re-diversion in salvage urethrectomy. ***a,*** *The apex and the proximal side of the appendix is anastomosed to the abdominal wall and neobladder, respectively.* ***b,*** *Windows are created in the meso-appendix, seromuscular sutures are placed through the meso-appendix as a subserosal tunnel.* ***c,*** *Conversion to an ileal conduit using the afferent limb of the Studer pouch. Isolated ileum was interposed between the afferent limb and the left side of the abdominal wall, because the afferent limb was too short to reach the abdominal wall.*

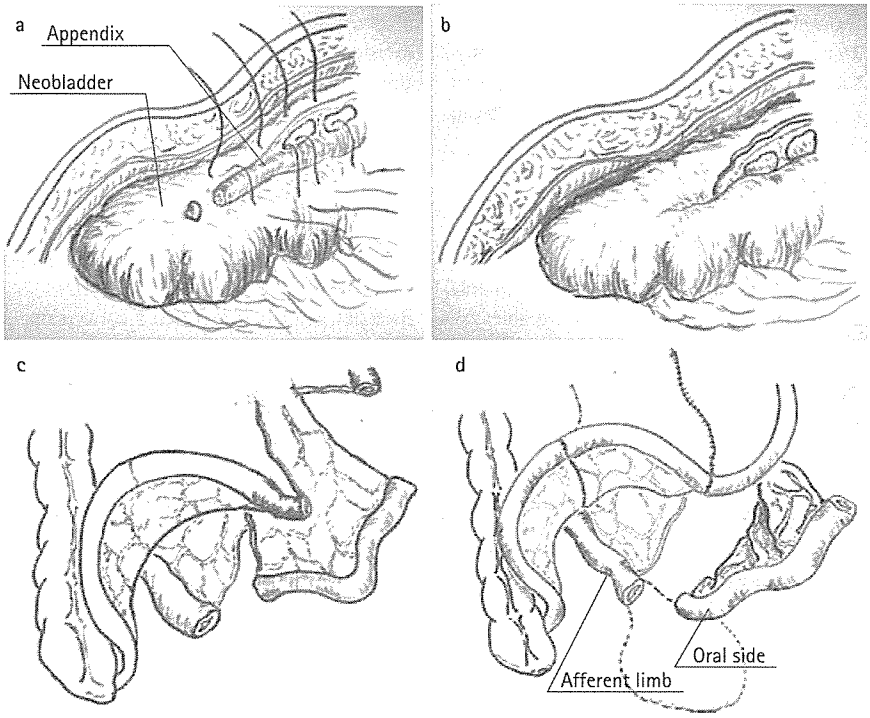

TABLE 1 Patients with urethral recurrence after an ileal neobladder

	Patient			
Variable	1	2	3	4
Age, years/gender	61/M	59/M	70/M	69/M
Radical cystectomy:				
P stage, grade	pT1b G2	pT2 G2	pT3a G2	pT1b G2
Type of pouch	Hautmann	Hautmann	Hautmann	Studer
First recurrence:				
time from cystectomy, months	45	6	17	39
P stage, grade	pTa G1	pTa G3	pTa G3	pTa G3
Subsequent recurrence:				
time from cystectomy, months	–	13	30, 34, 44	68
therapy	–	Urethrectomy*	TUR × 2 Urethrectomy*	Urethrectomy†
P stage/grade	–	pT1 G3	pTa G3 × 3	pT1 G3
Follow-up:				
status	NED	NED	NED	NED
time from cystectomy, months	89	30	61	70

*NED, no evidence of disease; *conversion to a cutaneous continent reservoir using the appendix; †conversion to an ileal conduit using the afferent limb of the Studer pouch.*

other was the conversion from a Studer pouch [6] to an ileal conduit, as reported previously (Fig. 1c) [3].

RESULTS

Of the four patients (5%) who had a urethral recurrence, three were in a Hautmann and one in a Studer pouch (Table 1). In all four patients, only positive cytology in voided urine samples led to the diagnosis of urethral recurrence, with no other symptoms. All cases were papillary pedunculate tumours in the penile urethra and initially treated with TUR. Radiological examination showed no evidence of metastasis or lymph node swelling in any of the four patients. The intervals between radical cystectomy and urethral recurrence were 6, 17, 39 and 45 months. The pathological findings of the urethral tumours at the first recurrence were pTa, with complete resection in all four cases.

During the follow-up, one patient (no. 1) showed no recurrence at 45 months from the initial TUR. However, the other three patients had further urethral recurrence and subsequently were treated by salvage urethrectomy. For urinary re-diversion two patients with a Hautmann pouch (nos. 2 and 3) chose conversion to a continent reservoir, and the one with a Studer pouch (no. 4) an ileal conduit. In the first two patients the reservoir was not excised and the apex of the appendix was anastomosed to the wall of the reservoir with a subserosal tunnel. Windows were created in the meso-appendix and seromuscular sutures placed through these windows to maintain a good blood supply. We made a stoma from the proximal side of the appendix (Fig. 1a,b). In the patient with the Studer pouch, the reservoir was excised and converted to an ileal conduit using the afferent limb. An isolated ileal segment of 5 cm was interposed between the afferent limb and abdominal wall, because the afferent limb was tightly attached to the retroperitoneum and too short to make a stoma on the abdominal wall (Fig. 1c). The operative duration was 625 min (no. 1), 535 min (no. 2) and 755 min (no. 3). There were no serious complications during surgery and the subsequent course was uneventful. The capacity of the continent reservoir was 400 mL at 1 month after surgery. The pathological stage of the urethral tumour at urethrectomy was pTa (no. 3), and pT1 (no. 2 and 4). There was no evidence of metastasis or

local recurrence in any of the four patients during the follow-up.

DISCUSSION

Urethral recurrence is a critical problem in neobladder reconstruction; the risk factors include multifocality, bladder neck involvement, carcinoma *in situ* in the bladder, and involvement in the upper urinary tract, bladder neck, prostatic urethra or prostate [1,2]. The rate of urethral recurrence after radical cystectomy with neobladder reconstruction is 2–6% [1–3]. Recently, Hassan *et al.* [7] reported a much lower rate (0.5%) of urethral recurrence when patients with obvious risk factors were excluded. In the present series, four patients (5%) had a urethral recurrence of urothelial carcinoma after neobladder reconstruction, although we did not substitute the neobladder in patients with relatively strict criteria, as described previously.

It was reported that the prognosis for urethral recurrence is poor, the cause possibly being related to the anatomical structure of the urethra, in which the lamina propria is the only barrier between the urethral mucosa and the vascular corpora [8]. Schellhammer and Whitmore [9] reported that 21 of 24 patients with urethral recurrence died within 5 years of surgery. In the series by Poole-Wilson and Barnard [10], all 14 patients with urethral recurrence died within 20 months. Recently, Clark *et al.* [4] showed that for patients with urethral recurrence after cystectomy, the urethral TCC stage was the most important predictor of overall survival, with the median survival for superficial (pTa or pTis) being 58.5% and for invasive (≥pT1) being 17.1%.

By contrast with the therapy for invasive urethral recurrence, there is controversy about the management of superficial urethral recurrence. Preservation of the urethra can maintain a high quality of life, and several investigators recently tried to preserve the urethra by conservative therapy. Leissner *et al.* [11] treated two cases of urethral recurrence (pTaG3, pT1G3) by TUR and both patients were tumour-free for 59 months. Yossepowitch *et al.* [12] reported that two urethral recurrences in patients with a neobladder were treated by TUR and both were free of disease by the last follow-up (the period being uncertain); one was treated by chemotherapy because of advanced disease. Studer and Zingg [6] reported seven patients with a urethral recurrence, of whom two were treated by urethrectomy and five conservatively. Miller and Benson [13] reported a patient with a neobladder and multiple urethral recurrences, treated with urethral resection and fulguration. The patient showed no evidence of recurrence for 14 months after resection. Several reports suggested that intraurethral instillation with 5-fluorouracil or BCG contributed successfully to preserving the urethra [2–4,14].

In the present series, all four urethral recurrences were superficial (pTa) and we attempted to preserve the urethra by TUR alone at the first recurrence. However, in only one of these four patients were we able to preserve the urethra, while the other three were finally treated with salvage urethrectomy due to further urethral recurrences. The pathological stage of urethral tumour at urethrectomy advanced from pTa to pT1 and pT2 in two of the three patients. This suggests that TUR alone is insufficient to control urethral recurrences. If there is further urethral recurrence then salvage urethrectomy should be used. Considering that all four patients are alive with no metastasis, preservation at initial therapy for superficial recurrence might be reasonable and sequential urethrectomy after attempted urethral preservation might be strategically feasible. Some additional therapy, e.g. BCG or 5-fluorouracil instillation, together with TUR might be effective in suppressing urethral recurrence.

Urethral recurrence was reportedly identified by several symptoms, or on cytology. Initial symptoms for urethral recurrence were obstructive LUTS, macrohaematuria, penile pain or mass, and urinary incontinence [3,15]. Urinary cytology is also reported to be useful for detecting urethral recurrence [16]. Clark *et al.* [4] reported that urinary cytology alone still detects a significant proportion of recurrences. In the present series, positive cytology led to the detection of urethral recurrence in all four patients, who had no symptoms. As the prognosis of superficial urethral recurrence is better than that of invasive recurrence, early detection is needed for such urethral recurrence. Thus, a noninvasive examination, e.g. urinary cytology, should be included as a part of the routine follow-up procedure, and urethroscopy used in patients presenting with positive cytology or symptoms.

Several techniques are reported for urinary re-diversion at urethrectomy for patients with an ileal neobladder; these are classified into two categories, the incontinent type (e.g. ileal conduit) and conversion to a continent reservoir. Huguet *et al.* [3] reported that the afferent limb was used as an ileal conduit after urethrectomy and partial neobladder excision, in a patient with a Studer pouch. In the present patient with a Studer pouch, the pouch was excised and converted to an ileal conduit using the afferent limb. However, the afferent limb was too short, being restricted with tight adhesions, to make a stoma on the abdominal wall, and thus an additional 15 cm-long ileal segment was interposed. As for conversion to a continent reservoir, Bartolleti *et al.* [17] reported a conversion from a Studer pouch to a continent urinary diversion, using an isolated 20-cm ileal loop which was invaginated for 10 cm. In the present series, we converted the Hautmann pouch into a continent reservoir using the appendix, similar to the Appe-Mainz procedure. The appendix has been used often for urinary diversion, e.g. interposition in the ureter, the Appe-Mainz procedure, etc. However, to our knowledge, conversion from an ileal neobladder to a continent reservoir using the appendix has not been reported previously. As the wall of the ileum (neobladder) is thinner than that of the colon or bladder, we anastomosed the thin side (apex) of the appendix, to maintain a good blood supply to the appendix, when creating the seromuscular tunnel. Neither of the patients had any complications after surgery.

In conclusion, urethral preservation as the initial therapy for superficial recurrence might be reasonable, and sequential urethrectomy after attempted urethral preservation might be strategically feasible. Urinary re-diversion from a neobladder to a catheterizable continent reservoir using the appendix would be a good choice and should maintain the patient's quality of life.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **TUR**, transurethral resection.

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Cancer Genetics Report

Association of the *PIG3* Promoter Polymorphism with Invasive Bladder Cancer in a Japanese Population

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PIG3 (p53-induced gene 3) is one of the targets of TP53 and is involved in apoptosis. The promoter of *PIG3* contains a variable number of tandem repeats (VNTRs) of pentanucleotides (TGYCC)*n* (Y = C or T) and the number of VNTRs was reported to be correlated with the activation by TP53. In this study, the clinical significance of the *PIG3* promoter VNTRs was analyzed in the bladder cancer patients using the genome DNAs from 338 controls and 273 bladder cancer patients. There was no significant difference in the allele frequency of the *PIG3* promoter VNTRs between them. However, the presence of 14 or less repeats allele was associated with higher cancer grade ($P = 0.038$) and higher stage in relative risk (adjusted odds ratio = 2.31, 95% confidence interval = 1.05–5.90). These data suggested that the *PIG3* promoter VNTRs was associated with generation of invasive bladder cancer.

Key words: PIG3 – bladder cancer – VNTRs

INTRODUCTION

Bladder cancer is the 5th most common malignancy in males in Western society, and the 10th most common cause of cancer death (1). In Japan, it is the 12th most common cancer in males and approximately 1.5 people per 1 000 000 die of the disease annually (2,3). Histologically, 90% of cases present transitional cell carcinoma (TCC), and >70% of the bladder TCC present as the superficial and papillary subtype (4,5). They are usually treated with endoscopic transurethral resection (TUR), although >50% of them suffer recurrences in the bladder 5 years after TUR and 5–20% of them progress to life-threatening muscle invasive TCC (4,5). Invasive TCCs are highly aggressive and about half of them will recur with distant metastasis even if radical treatment is performed.

Mutations in the p53 gene (*p53*), one of the best characterized tumor suppressor genes, are the most common genetic events in human malignancies, including the bladder TCC (6). Alterations in *p53* are linked to a high stage, a high grade and a poor prognosis of the bladder TCC (7–11). TP53 acts as a transcription factor and is involved in the regulation of cellular proliferation and apoptosis under genotoxic conditions (12). Recently, *PIG3* (p53-induced gene 3) was identified as a result of the serial analysis of gene expression using the colorectal cancer cell line DLD-1 overexpressing TP53, and was defined as one of the genes associated with apoptosis (13). Interestingly, the promoter region of *PIG3* contains a variable number of tandem repeats (VNTRs) of pentanucleotide sequences (TGYCC)*n* (Y = T or C), which were directly bound to by TP53, and the *PIG3* promoter VNTRs were reported to correlate with the activation by TP53 *in vitro* (14). The previous data that PIG3 was related with apoptosis in the cancer cells provided the hypothesis that possession of the short allele of the *PIG3* promoter was associated with susceptibility to cancers because of its reduced activation by TP53. In this study, we explored the association between the *PIG3* promoter VNTRs, and the susceptibility and disease status of the bladder TCC.

SUBJECTS AND METHODS

SUBJECTS

A total of 273 patients with the bladder TCC who were treated at Kyoto University Hospital in Kyoto Prefecture between June 1990 and December 2002 were enrolled in this study. All materials from the patients which have been preserved were

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