Evaluation for Surrogacy of End Points by Using Data from Observational Studies: Tumor Downstaging for Evaluating Neoadjuvant Chemotherapy in Invasive Bladder Cancer

Satoshi Teramukai,[1] Hiroyuki Nishiyama,[2] Yoshiyuki Matsui,[2] Osamu Ogawa,[2] and Masanori Fukushima[1]

Abstract **Purpose:** In clinical cancer trials for evaluating neoadjuvant chemotherapy, tumor downstaging is frequently used as a surrogate end point for overall survival. We evaluated the surrogacy of tumor downstaging using data from a follow-up observational study in bladder cancer.

Experimental Design: A total of 586 patients (from 32 Japanese hospitals) who underwent radical cystectomy for invasive bladder cancer (clinical T2 to T4) between 1990 and 2000 were analyzed. We considered changes over time in clinical stage at diagnosis and pathologic stage at cystectomy as a surrogate end point, and survival time after cystectomy as a true end point. First, we developed a new criterion for tumor downstaging. Second, we statistically evaluated surrogacy for the criterion using Prentice's criteria.

Results: To develop the criterion of end points based on tumor downstaging, we selected the best classification among all possible classifications in an attempt to separate prognosis for patients. The hazard ratios after adjustment for prognostic factors in the intermediate effect patients and the poor effect patients were 1.9 (95% confidence interval, 1.0-3.7) and 5.0 (95% confidence interval, 2.6-9.8), respectively, compared with that in the good effect patients. The conditions for correlation and conditional independency of Prentice's criteria were satisfied approximately. Neoadjuvant chemotherapy has a statistically significant tumor downstaging effect, whereas there was no difference on survival between treatment groups.

Conclusions: The tumor downstaging effect could be an appropriate intermediate end point for screening novel neoadjuvant chemotherapy for invasive bladder cancer. The dataset from follow-up studies were useful for evaluating the surrogacy of end points.

Appropriate surrogate end points are critical for developing new therapies through evaluation of biological activity. The surrogate end point is a test, measurement, score, or some other similar variable that is used in place of a clinical event in the design of a trial, or in summarizing results from it. Used because the variable is believed to be correlated with the clinical event of interest and because of its perceived utility in yielding detectable treatment differences (1). In clinical cancer trials, overall survival is considered to be the most reliable and definitive true end point. However, surrogate end points such as tumor burden outcomes including objective tumor effect, disease-free survival, and progression-free survival, or biomarkers including prostate-specific antigen have been widely used because trials with the true clinical outcome are often longer and larger. In a recent analysis for oncologic drugs in the U.S., 68% (39 of 57) of the regular approvals and all of the 14 accelerated approvals were based on end points other than overall survival in the last 13 years (2). To use a valid and reliable surrogate end point in cancer clinical trials, we should evaluate the surrogacy of end points on a case-by-case basis because the adequacy as a surrogate end point is highly dependent upon the type and/or stage of cancer, and other available therapies.

For statistical validation of surrogate end points, Prentice (3) proposed the validity criterion that a valid between-group analysis of the surrogate end point also constitutes a valid analysis of the true clinical end point. Freedman et al. (4) showed that these criteria were not straightforward to verify by hypothesis testing. Recently, Buyse et al. (5) have proposed two new measures, termed "relative effect" and "adjusted association." However, to explore the validity of a surrogate end point by these measures, we have to combine information from several randomized clinical trials testing the effect of a treatment on both the surrogate and the true end points (6). In practice, we rarely have information about both end points from even single randomized clinical trials before designing a feature clinical trial for new agents. Such situations have motivated us to assess the surrogacy of end points using

Authors' Affiliations: [1]Department of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital and [2]Department of Urology, Kyoto University Graduate School of Medicine, Kyoto, Japan

Received 7/24/05; revised 9/17/05; accepted 10/10/05.

Grant support: Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, and Technology of Japan.

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Requests for reprints: Satoshi Teramukai, Department of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, 54 Shogoin Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-4768; Fax: 81-75-751-4732; E-mail: steramu@kuhp.kyoto-u.ac.jp.

doi:10.1158/1078-0432.CCR-05-1598

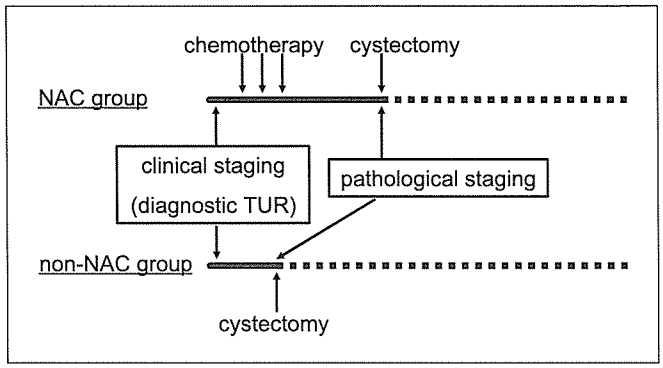

Fig. 1. Schema of treatment group comparison.

available information other than randomized studies. In clinical trials for evaluating neoadjuvant chemotherapy in bladder cancer, "tumor downstaging" is frequently used as a surrogate end point for overall survival. Clinical staging with transurethral resection (TUR) is very important in treatment planning and prognosis. However, the reliability of TUR staging is a problem. The disparity between clinical and pathologic staging may be caused by repeat TUR, i.e., TUR effect, and measurement error (7). We developed a new criterion of tumor downstaging effect and evaluated the surrogacy of tumor downstaging using data from a follow-up observational study in invasive bladder cancer.

Patients and Methods

A total of 1,131 patients who underwent radical cystectomy for invasive bladder cancer between 1990 and 2000 at 32 Japanese hospitals were retrospectively registered (8). The information that was collected from the medical records included age, gender, histology, clinical staging, and pathologic staging according to the tumor-node-metastasis classification (9), and the presence of perioperative systemic chemotherapy. In the present study, 586 patients who have clinical stage T2 to T4, N0, M0, transitional cell carcinoma, and who were less than 80 years old were included.

Figure 1 shows a schema of treatment group comparison. The patients were divided into two treatment groups, i.e., neoadjuvant chemotherapy (NAC) group and no neoadjuvant chemotherapy (non-NAC) group. After the clinical staging was done based on diagnostic TUR, chemotherapy followed by radical cystectomy was done in the NAC group, and only cystectomy was done in the non-NAC group. More precise pathologic staging was done at the time of cystectomy.

Statistical analysis. Prentice's criterion for evaluating the surrogacy of end points is a set of four conditions as follows (3, 5, 10):

PC1: $f(T|Z) \neq f(T)$ so the treatment affects the distribution of T,
PC2: $f(S|Z) \neq f(S)$ so the treatment affects the distribution of S,
PC3: $f(T|S) \neq f(T)$ so the surrogate affects the distribution of T,
PC4: $f(T|S,Z) = f(T|S)$ so that conditionally on S, T is independent of Z.

where, for example, $f(T|Z)$ is the conditional distribution of the true end point T given the treatment assignment Z, and S is the surrogate end point. In the present study, the treatment Z is set to 0 for non-NAC group and 1 for NAC group. The candidate surrogate end point S is a tumor downstaging effect based on the difference between clinical stage and pathologic stage and the true end point T is overall survival after cystectomy. Therefore, in this setting, the PC1 means that neoadjuvant chemotherapy must affect overall survival, PC2 means that neoadjuvant chemotherapy must affect tumor downstaging, PC3 means that tumor downstaging must be correlated with overall survival, and PC4 means that tumor downstaging must fully capture the net effect of neoadjuvant chemotherapy on overall survival.

The survival curves were estimated with the Kaplan-Meier method. The Cox proportional hazards model was used to estimate hazard ratios (HR) after adjustment for covariates. All statistical analyses were done by using SAS version 8.02 (SAS Institute, Inc., Cary, NC).

Results

A total of 586 patients [481 men (82%) and 105 women (18%)], with a mean age of 65.2 years (range, 33-80 years), were treated with radical cystectomy with bilateral lymph node dissection. Out of 586 patients, 183 patients (31%) were treated with neoadjuvant chemotherapy. As the neoadjuvant chemotherapy, methotrexate, vinblastine, doxorubicin, and cisplatin, was used in 43% of patients and used for 1.5 cycles on average. The other patients were treated with the modified cisplatin-based regimens including methotrexate, epirubicin and cisplatin; and cisplatin, cyclophosphamide, and doxorubicin; and cisplatin, adriamycin, and methotrexate, as well as other miscellaneous regimens (11-15). The distributions of prognostic factors in treatment groups were as follows: mean patient age was 65.8 years (SD, 8.8) and 63.7 years (SD, 8.6)

Table 1. Hazard ratios by clinical stage and pathologic stage

Clinical stage	Pathologic stage (95% CI)				
	P0/1	P2a	P2b	P3	P4
All cases					
T2	1	1.9 (0.9-4.1)	2.4 (0.9-6.1)	4.3 (1.8-10.3)	11.1 (4.2-29.5)
T3/4	1.5 (0.6-3.6)	2.2 (0.9-5.5)	4.6 (2.2-9.7)	5.3 (2.6-10.7)	5.3 (2.5-11.6)
Non-NAC group					
T2	1, $n = 59$	2.2 (0.9-5.6), $n = 81$	2.7 (0.9-8.0), $n = 30$	4.9 (1.7-14.0), $n = 22$	14.4 (4.5-45.9), $n = 11$
T3/4	2.6 (0.8-8.5), $n = 26$	2.2 (0.7-7.1), $n = 24$	5.5 (2.1-14.1), $n = 43$	6.2 (2.5-15.3), $n = 80$	5.3 (1.9-14.7), $n = 27$
NAC group					
T2	1, $n = 27$	1.3 (0.3-6.0), $n = 18$	2.6 (0.3-23.5), $n = 3$	3.4 (0.6-21.1), $n = 4$	11.0 (1.2-103), $n = 2$
T3/4	1.0 (0.3-3.5), $n = 40$	2.5 (0.6-10.4), $n = 13$	3.7 (1.1-12.3), $n = 20$	3.9 (1.2-12.2), $n = 40$	5.5 (1.7-18.2), $n = 16$

in the non-NAC and NAC groups, respectively. The patient proportion of positive lymph node involvement was slightly higher in the non-NAC group (17.4%) than in the NAC group (14.2%), but that of clinical T3 or T4 was much higher in the NAC group (70.5%) than in non-NAC group (49.6%). Proportions of receiving postoperative chemotherapy were similar in both groups, i.e., 23.1% in the non-NAC group, 23.0% in the NAC group.

Development of tumor downstaging effect criterion. We estimated HRs on the overall survival after cystectomy by 10 combinations of clinical and pathologic stage after adjustment for age, lymph node involvement, and adjuvant chemotherapy (Table 1). The estimated HRs by treatment group were similar to that in all cases. First, the 10 combinations were ordered according to the size of HR [1, T2 to P0/1 (HR, 1); 2, T3/4 to P0/1 (HR, 1.5); 3, T2 to P2a (HR, 1.9); 4, T3/4 to P2a (HR, 2.2); 5, T2 to P2b (HR, 2.4); 6, T2 to P3 (HR, 4.3); 7, T3/4 to P2b (HR, 4.6); 8, T3/4 to P3 (HR, 5.3); 9, T3/4 to P4 (HR, 5.3); 10, T2 to P4 (HR, 11.1)] in all cases. Second, we selected the best classification among all possible classifications in an attempt to separate the prognosis of patients with respect to the Akaike's information criteria. The total number of examined classifications was 45—9 for two categories (good/poor) and 36 for three categories (good/intermediate/poor). For example, the examined classifications were 1 (good) versus 2 to 10 (poor), 1 to 2 versus 3 to 10,..., 1 to 9 versus 10 for two categories, and 1 (poor) versus 2 (intermediate) versus 3 to 10 (poor), 1 versus 2 to 3 versus 4 to 10, 1 versus 2 to 4 versus 5 to 10,..., 1 to 8 versus 9 versus 10 for three categories.

As a result, patients were classified into three categories, i.e., good effect (1, T2 to P0/1), intermediate effect (2-5, T2 to P2a/2b or T3/4 to P0/1/2a), and poor effect (6-10, T2 to P3/4 or T3/4 to P2b/3/4). Survival curves according to the tumor downstaging effect were shown in Fig. 2A. The HRs in the intermediate effect patients and the poor effect patients were 1.9 [95% confidence interval (CI), 1.0-3.7] and 5.0 (95% CI, 2.6-9.8), respectively, compared with that in the good effect patients after adjustment for age, lymph node involvement, and adjuvant chemotherapy. The risks by tumor downstaging effect were similar between treatment groups (Fig. 2B and C).

Statistical evaluation for surrogacy of the end point. It is obvious that to fulfill the PC3 condition, tumor downstaging must be correlated with overall survival because we selected the tumor downstaging in such a way that the patients can be classified based on their overall survival. To verify the PC4 condition that tumor downstaging must fully capture the net effect of neoadjuvant chemotherapy on overall survival, it is usually stated that the coefficient corresponding to treatment effect corrected for tumor downstaging is required to be equal to zero. The HRs between treatment groups by tumor downstaging effect, pooled HR and their 95% CIs were estimated after adjustment for age, lymph node involvement, and adjuvant chemotherapy (Table 2). The estimated pooled HR was 1.06 (95% CI, 0.77-1.47) when stratifying by tumor downstaging effect. Although the nonsignificance of the test in which HR = 1 does not prove the PC4 condition, it was suggested that PC4 might be plausible in this study because the pooled HR was close to 1.

As the data is not from randomized trials, strictly speaking, the inference for treatment comparison is not valid and thus

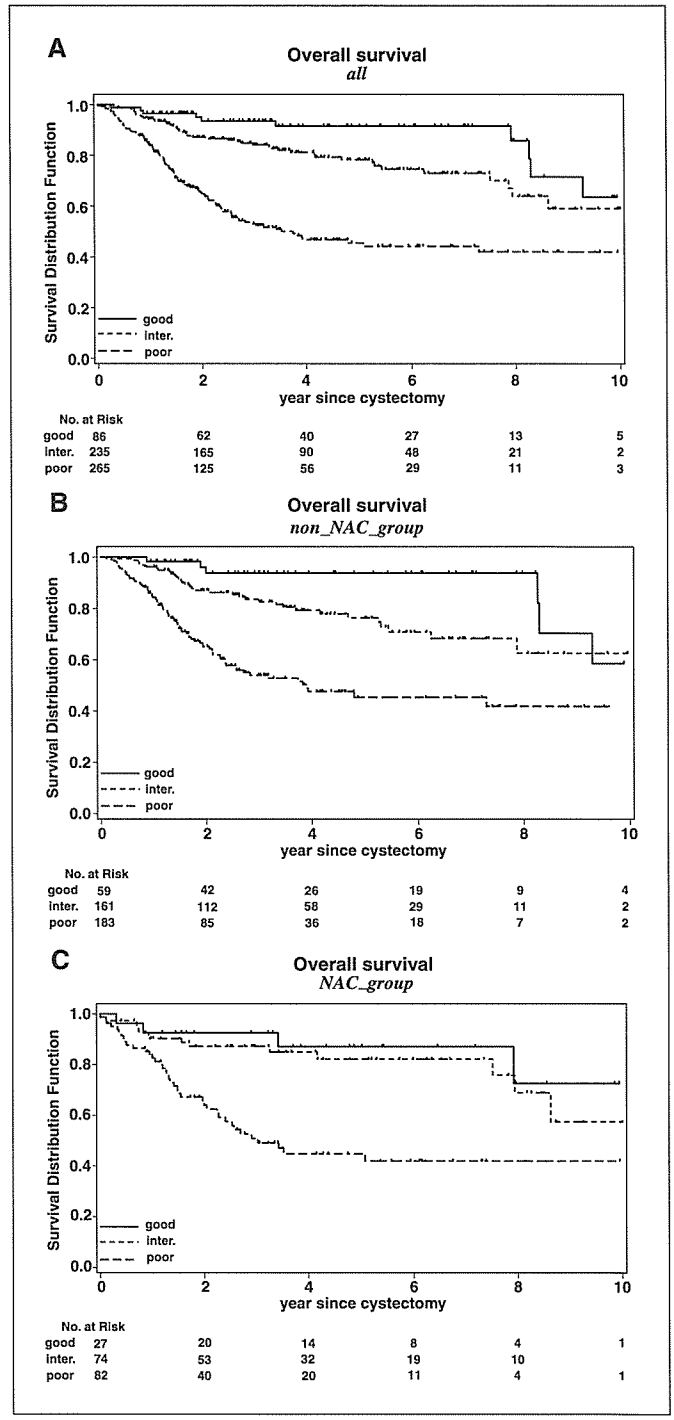

Fig. 2. Survival curves according to tumor downstaging effect in all patients (*A*), in the nonneoadjuvant chemotherapy group (*B*), and in the neoadjuvant chemotherapy group (*C*).

the PC1 and PC2 conditions cannot be evaluated. However, we attempted to verify the PC1 and PC2 conditions after adjustment for the confounding factors. For evaluating PC2, we used the Cochran-Mantel-Haenszel statistic with rank score, i.e., the stratum-adjusted Wilcoxon test, because of imbalance of clinical stage distribution among treatment groups. The effect of neoadjuvant chemotherapy on tumor downstaging effect was statistically significant (χ^2 = 16.1, P = 0.001; Table 3).

Table 2. Overall survival HRs for NAC over non-NAC, according to tumor downstaging effect

Tumor downstaging effect	No. of patients		HR (95% CI)
	NAC	Non-NAC	
Good	27 (15%)	59 (15%)	1.32 (0.37-4.74)
Intermediate	74 (40%)	161 (40%)	0.76 (0.40-1.45)
Poor	82 (45%)	183 (45%)	1.17 (0.79-1.73)
Pooled, stratified by tumor downstaging effect	183	403	1.06 (0.77-1.47)

To evaluate the PC1 condition, we compared the overall survival between treatment groups by clinical stage. In clinical stage T2, the treatment effect was not statistically significant (HR, 0.87; 95% CI, 0.44-1.70) after adjustment for age, lymph node involvement, and adjuvant chemotherapy. Similarly, in clinical stage T3 or T4, the treatment effect was not statistically significant (HR, 0.98; 95% CI, 0.67-1.43).

Discussion

In this study, we proposed a new tumor downstaging criterion based on prognosis in invasive bladder cancer patients for evaluating neoadjuvant chemotherapy. Objective tumor response has been a widely accepted measure of cancer chemotherapy activity. According to international standards, including WHO criteria (16) and Response Evaluation Criteria in Solid Tumors (17), patients were usually classified into either responders (complete response or partial response) or non-responders (no change or progressive disease). The objective tumor response can be assessed even in single-arm studies, however, in the NAC group of the present study, overall survival had no difference between responders and non-responders for neoadjuvant chemotherapy (adjusted HR, 1.09; 95% CI, 0.59-2.03; Fig. 3). Therefore, objective tumor response might not be a valid surrogate end point for evaluating neoadjuvant chemotherapy in invasive bladder cancer.

Some investigators defined the criterion for tumor downstaging (7, 18). However, few data were available with regard to clinical staging and pathologic staging for patients who were treated with or without neoadjuvant chemotherapy, and no definite criterion has been developed based on the prognosis of patients. In the present study, the HRs among clinical stages were different even on the same pathologic stage, especially on $P_{0/1}$ and P_{2b} in the non-NAC group (Table 1). This suggests that unmeasurable components, including the clinician's subjective judgment on clinical stage, might reflect different prognoses. With regard to tumor downstaging in invasive bladder cancer, it is questionable to generalize the findings to other cancers because downstaging can occur without chemotherapy when the tumor is removed by the diagnostic TUR (7). In addition to the TUR effect, misclassification for staging system, called staging error, have to be considered. In the present study, a proportion of good downstaging effect was 29% even in the non-NAC group. This means that a control group is essential for evaluating therapies in invasive bladder cancer if the tumor downstaging effect is used as an end point of clinical trials.

We statistically evaluated the surrogacy of the end point using data from a follow-up observational study. Prentice's criterion was useful for that purpose, especially for the evaluation of PC3 (correlation) and PC4 (conditional independency). In the present study, the PC3 and PC4 conditions were satisfied approximately. Although the study is not a randomized trial, it is suggested that the neoadjuvant chemotherapy affects tumor downstaging, i.e., PC2 (tumor downstaging benefit) is acceptable, but the treatment does not affect overall survival, i.e., PC1 (survival benefit) is unacceptable. We gave an actual example of hypothetical situations from other articles (5, 10), which showed that the PC2 does not imply the PC1. As another actual case, a randomized trial for locally advanced bladder cancer concluded that the survival benefit of neoadjuvant chemotherapy was of borderline statistical significance ($P = 0.06$), whereas the tumor downstaging effect was statistically significant ($P = 0.001$; ref. 7). Do the inconsistent results between PC1 and PC2 depend on the differences of statistical power for evaluating these conditions? We calculated the power of two kinds of statistical tests, i.e., Wilcoxon rank-sum test for tumor downstaging effect and log-rank test for overall survival, based on our data. If the expected proportions of downstaging effect are 0.50 (good), 0.39 (intermediate), and 0.11 (poor) in the NAC group and 0.29 (good), 0.55 (intermediate), and 0.16 (poor) in the non-NAC group from the data in clinical stage T2, a sample size of 96 in each group will have 80% power to reject the null hypothesis using a Wilcoxon rank-sum test with a 0.05 two-sided significance level (19). On the other hand, if the expected 5-year survival probability in the non-NAC group is 0.5, 0.6, and 0.7 and HR is 0.87, a corresponding sample size in each group will be 1,595, 2,004, and 2,683, respectively, using a 0.05 level two-sided log-rank test for equality of survival curves (20). The difference of statistical power is critical for evaluating the PC1 and PC2 conditions. In two recently published studies, the survival curves for patients treated with neoadjuvant

Table 3. Tumor downstaging effect of treatment according to clinical stage

Clinical stage	Treatment	Tumor downstaging effect			Total
		Good	Intermediate	Poor	
T2	NAC	27 (50%)	21 (39%)	6 (11%)	54
	non-NAC	59 (29%)	111 (55%)	33 (16%)	203
T3/4	NAC	0	53 (41%)	76 (59%)	129
	non-NAC	0	50 (25%)	150 (75%)	200

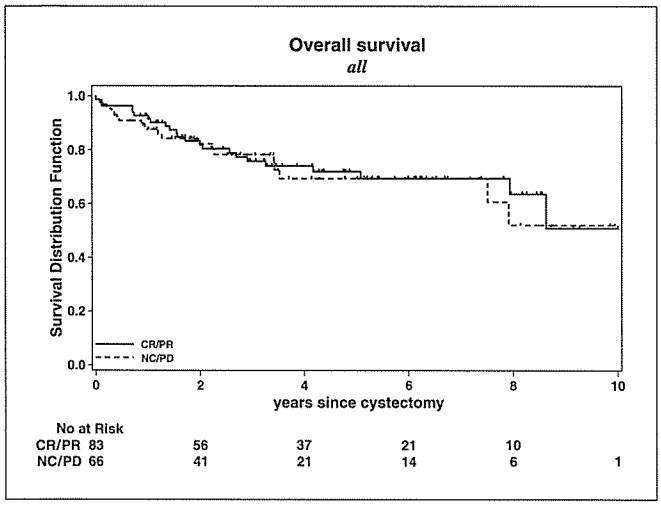

Fig. 3. Survival curves according to tumor response (CR/PR versus NC/PD). CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

methotrexate, vinblastine, doxorubicin, and cisplatin was superior for patients treated with cystectomy alone, with a HR of 0.74 (95% CI, 0.55-0.99) in a randomized trial (7, 21), and platinum-based combination chemotherapy showed a survival benefit with a HR of 0.87 (95% CI, 0.78-0.97) in a meta-analysis of individual patient data (22). The HR which we assumed to calculate the power might be plausible from these results. However, an important question for implementing neoadjuvant chemotherapy for patients with invasive bladder cancer remains, i.e., how do we select the appropriate patients for combination therapy (23).

Buyse et al. (5, 6) have emphasized that we have to combine information from several randomized clinical trials testing the effects of treatment on both surrogate and true end points to explore the validity of a surrogate end point. In practice, we must assess the surrogacy of a candidate end point without data from a randomized trial because the primary objective of a randomized trial will often be to evaluate survival benefit, hence, if the survival benefit were known to be true, then one would have to question the value of conducting such a study. Nonetheless, the purpose of the evaluation of surrogacy should be restricted to find out "appropriate intermediate end points" (10). Fleming et al. (24) also pointed out that surrogate end points can be useful in phase 2 screening trials for identifying whether a new intervention is biologically active and for guiding decisions about whether the intervention is promising enough to justify a large definitive trial with clinically meaningful outcomes. The basic premise is that we cannot predict a treatment effect on the true end point from the effect on the surrogate end point. In conclusion, the tumor downstaging effect could be an appropriate intermediate end point in phase 2 trials for screening novel neoadjuvant chemotherapy in invasive bladder cancer. The dataset from follow-up studies were useful for evaluating the surrogacy of end points.

Acknowledgments

We thank the hospitals associated with Kyoto University, Nara Medical University, and Nagoya University for providing clinical data.

References

1. Meinert CL. Clinical trials: design, conduct, and analysis. Oxford: Oxford University Press; 1986. p. 304.
2. Johnson JR, Williams G, Pazdur R. End points and United States Food and Drug Administration approval of oncology drugs. J Clin Oncol 2003;21:1404–11.
3. Prentice RL. Surrogate end points in clinical trials: definition and operational criteria. Stat Med 1989;8: 431–40.
4. Freedman LS, Graubard BI, Schatzkin A. Statistical validation of intermediate endpoints for chronic diseases. Stat Med 1992;11:167–78.
5. Buyse M, Molenberghs G. Criteria for the validation of surrogate endpoints in randomized experiments. Biometrics 1998;54:1014–29.
6. Buyse M, Molenberghs G, Burzykowski T, Renard D, Geys H. The validation of surrogate endpoints in meta-analyses of randomized experiments. Biostatistics 2000;1:49–67.
7. Grossman HB, Natale RB, Tangen CM, et al. Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. N Engl J Med 2003;349:859–66.
8. Nishiyama H, Habuchi T, Watanabe J, et al. Clinical outcome of a large-scale multi-institutional retrospective study for locally advanced bladder cancer: a survey including 1131 patients treated during 1990–2000 in Japan. Eur Urol 2004;45:176–81.
9. Fleming ID, Cooper JS, Henson DE, et al., editors. AJCC cancer staging manual. 5th ed. Philadelphia: Lippincott-Raven; 1997. p. 241–3.
10. Berger VW. Does the Prentice criterion validate surrogate endpoints? Stat Med 2004;23:1571–8.
11. Sternberg CN, Yagoda A, Scher HI, et al. Preliminary results of M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin) for transitional cell carcinoma of the urothelium. J Urol 1985;133:403–7.
12. Kuroda M, Kotake T, Akaza H, Hinotsu S, Kakizoe T. Efficacy of dose-intensified MEC (methotrexate, epirubicin and cisplatin) chemotherapy for advanced urothelial carcinoma: a prospective randomized trial comparing MEC and M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin). Jpn J Clin Oncol 1998;28:497–501.
13. Sternberg JJ, Bracken RB, Handel PB, Johnson DE. Combination chemotherapy (CISCA) for advanced urinary tract carcinoma. A preliminary report. JAMA 1977;238:2282–7.
14. Oshima S, Ono Y, Fujita T, et al. Three-drug combination chemotherapy for advanced urothelial tract carcinoma. Cancer Chemother Pharmacol 1987;20: S20–3.
15. Matsui Y, Nishiyama H, Watanabe J, et al. The current status of perioperative chemotherapy for invasive bladder cancer: a multiinstitutional retrospective study in Japan. Int J Clin Oncol 2005;10:133–8.
16. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. Cancer 1981; 47:207–14.
17. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 2000;92:205–16.
18. Splinter TAW, Scher HI, Denis L, et al. The prognostic value of the pathological response to combination chemotherapy before cystectomy in patients with invasive bladder cancer. J Urol 1992;147:606–8.
19. Kolassa JE. A comparison of size and power calculations for the Wilcoxon statistic for ordered categorical data. Stat Med 1995;14:1577–81.
20. Freedman LS. Tables of the number of patients required in clinical trials using the logrank test. Stat Med 1982;1:121–9.
21. Raghavan D. Chemotherapy and cystectomy for invasive transitional cell carcinoma of bladder. Urol Oncol 2003;21:468–74.
22. Advanced Bladder Cancer (ABC) Meta-analysis Collaboration. Neoadjuvant chemotherapy in invasive bladder cancer: a systematic review and meta-analysis. Lancet 2003;361:1927–34.
23. Millikan R, Siefker-Radtke A, Grossman HB. Neoadjuvant chemotherapy for bladder cancer. Urol Oncol 2003;21:464–7.
24. Fleming TR, DeMets DL. Surrogate end points in clinical trials: are we being misled? Ann Intern Med 1996;125:605–13.

International Journal of Urology (2006) **13,** 703–706

Original Article

5-year interval change in voiding function of orthotopic ileal neobladder

YOICHI ARAI[1], ATSUSHI TAKEDA[1], YOJI TAKI[2], YUSAKU OKADA[3] AND OSAMU OGAWA[4] AND THE TOHOKU-KYOTO URINARY RECONSTRUCTION STUDY GROUP*

[1]*Department of Urology, Tohoku University Graduate School of Medicine, Sendai,* [2]*Department of Urology, Toyooka Public Hospital, Toyooka,* [3]*Department of Urology, Shiga University of Medical Science, Otsu, and* [4]*Department of Urology, Kyoto University Graduate School of Medicine, Kyoto, Japan*

Aim: We report the 5-year interval change in voiding function of orthotopic ileal neobladder.
Methods: Voiding function was evaluated at two points with an interval of 5 years in 49 patients with orthotopic ileal neobladder. The first and second surveys were performed in May, 1998 (1998 survey) and in April 2003 (2003 survey), respectively. Median age at operation was 67 years, ranging 47–77. Median follow-up times at the first and the second surveys were 19.5 months (range, 3–87) and 67.5 months (range, 62–145), respectively.
Results: There was no significant change in daytime continence status between the 1998 and 2003 surveys. More than 95% never or only occasionally suffered daytime incontinence in the two surveys. On the other hand, 15 (34.1%) and 14 (31.8%), respectively, experienced night-time incontinence, despite regular voiding during the night. When voiding patterns were analysed, 11 patients (23.4%) sometimes or often performed catheterization because of difficulty in urinating or incomplete emptying of the neobladder in the 1998 survey. Three patients (6.4%) were unable to void and required regular catheterization. In the 2003 survey, however, such poor voiders increased to nine (19.1%), although the difference was not significant. During the study period of 5 years, there was no change in renal function.
Conclusions: 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. Careful long-term follow up is warranted.

Key words neobladder, urinary reconstruction, voiding function.

Introduction

The orthotopic neobladder enables patients to void through his or her own urethra. Thus, the neobladder is potentially capable of providing better quality of life for cystectomy patients. To date, many forms of bladder substitute have been reported, all giving comparable results.[1–5]

Correspondence: Yoichi Arai MD, Department of Urology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan.
Email: yarai@uro.med.tohoku.ac.jp

Received 5 September 2005; accepted 15 November 2005.
*Yoichi Arai, Atsushi Takeda, Masanori Kato; Tohoku University Graduate School of Medicine, Sendai. Yoji Taki, Hideo Takeuchi; Toyooka Public Hospital, Toyooka. Akito Tearai; Kurashiki Central Hospital, Kurashiki. Tatushiro Okabe; Shiga Medical Center for Adult Diseases, Moriyama. Teruo Konami; Otsu Municipal Hospital, Otsu. Mieko Miyakawa; Shimada Municipal Hospital, Shimada. Mutsushi Kawakita; Kobe City General Hospital, Kobe. Asaki Soeda; Nishi-Kobe Medical Center, Kobe. Yoshihiko Wakabayashi, Yusaku Okada; Shiga University of Medical Science, Otsu. Arata Higashi, Osamu Ogawa; Kyoto University Hospital, Kyoto.

There have been only a few reports on longitudinal functional outcome of neobladder creation.[6–9] We previously reported voiding function of ileal neobladder in both men and women.[6,7] The survey was performed in 1998. In 2003, 5 years after the initial survey, the second survey was performed for the same patients. We herein present the 5-year interval change in voiding function of orthotopic ileal neobladder.

Materials and methods

From November 1990 through February 1998, 71 patients underwent Hautmann bladder substitution in Kyoto University Hospital, Shiga University of Medical Science, and their affiliated hospitals. Among them, voiding function was evaluated at two points with an interval of 5 years in 49 patients, who comprised the study population. There were 43 men and six women, and median age at operation was 67 years old, ranging 47–77. Of these patients, 46 underwent simultaneous radical cystectomy. The three remaining patients underwent simultaneous unilateral nephroureterectomy and cystectomy for primary invasive ureteral cancer in the ureterovesical junction. All patients gave their full consent and were informed about other

options for urinary reconstruction. Patients were monitored at regular intervals of 3–6 months. Serum electrolytes, blood urea nitrogen and creatinine measurements, were performed at 3 months and then annually. Uroflowmetric study and postvoid residual urine measurements were performed arbitrarily.

The first and second surveys were performed in May, 1998 (1998 survey) and in April 2003 (2003 survey), respectively. Therefore, at the second survey, follow-up time was at least 5 years or longer. Median follow-up times at the first and the second surveys were 19.5 months (range, 3–87) and 67.5 months (range, 62–145), respectively. We questioned the patients regarding voiding behavior and continence, need for self-catheterization, voiding posture, incontinence, and the need for a pad to manage incontinence. If a patient wore a sanitary pad only for precautionary reasons or had only occasional spotting, he was regarded as continent. Statistical analyses were made using Wilcoxon or χ^2-test and $P < 0.05$ was considered statistically significant.

Results

The data on voiding function in the 1998 and 2003 surveys are shown in the Table 1. Since the study was retrospective, data were not available in some patients in each parameter. When voiding patterns were analysed, 11 patients (23.4%) sometimes or often performed catheterization because of difficulty in urinating or incomplete emptying of the neobladder in the 1998 survey. Three patients (6.4%) were unable to void and required regular catheterization. In the 2003 survey, however, nine (19.1%) were unable to void and needed regular catheterization, although the difference was not significant ($P = 0.174$). Of these nine patients, eight were male and one was female, thus there was no difference in the need for regular catheterization between the male and female: 18.6% and 16.7%, respectively. With regard to posture at voiding, 23 (52.3%) voided in a regular standing position in the 1998 survey. Eighteen patients (40.9%) preferred a sitting position while voiding, of whom 13 (29.5%) only voided in a sitting position. At the

Table 1 Voiding function of ileal neobladder patients

	1998 survey		2003 survey		*P*-value
	Number of patients	(%)	Number of patients	(%)	
Difficulty on urination†					0.174
None	28	(60.9)	25	(54.3)	
Yes	15	(32.6)	12	(26.1)	
Inability to void	3	(6.5)	9	(19.6)	
Need for catheterization†					0.301
Never	33	(70.2)	30	(63.8)	
Sometimes	5	(10.6)	4	(8.5)	
Often	6	(12.8)	4	(8.5)	
Routine catheterization	3	(6.4)	9	(19.1)	
Posture at voiding†					0.277
Standing position only	23	(52.3)	19	(43.2)	
Sitting position only	13	(29.5)	10	(22.7)	
Sitting position, sometimes	5	(11.4)	6	(13.6)	
Inability to void	3	(6.8)	9	(20.5)	
Daytime incontinence†					0.798
Never	38	(82.6)	37	(80.4)	
Occasionally	6	(13.0)	6	(13.0)	
Sometimes	0	(0.0)	1	(2.2)	
Always	2	(4.3)	2	(4.3)	
Night-time incontinence†					0.978
None	16	(36.4)	18	(40.9)	
None, if voided at night	13	(29.5)	12	(27.3)	
Yes, despite regular voiding at night	14	(31.8)	13	(29.5)	
Always	1	(2.3)	1	(2.3)	
Voiding frequency during night-time†					0.956
0	2	(5.6)	2	(5.6)	
1	18	(50.0)	19	(52.8)	
2	12	(33.3)	10	(27.8)	
3 or more	4	(11.1)	5	(13.9)	
Use of pads during daytime†					0.724
No	37	(90.2)	36	(87.8)	
Yes	4	(9.8)	5	(12.2)	
Use of pads during night-time†					0.499
No	21	(52.5)	24	(60.0)	
Yes	19	(47.5)	16	(40.0)	

†Data are not available in some patients.

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.

References

1 Hautmann RE, Egghart G, Frohneberg D, Miller K. The ileal neobladder. *J. Urol.* 1988; **139**: 39–42.
2 Elmajian DA, Stein JP, Esrig D *et al.* The Kock ileal neobladder: updated experience in 295 male patients. *J. Urol.* 1996; **156**: 920–5.
3 Reddy PK, Lange PH, Fraley EE. Total bladder replacement using detubularized sigmoid colon: technique and results. *J. Urol.* 1991; **145**: 51–5.
4 Flohr P, Hefty R, Paiss T, Hautmann RE. The ileal neobladder – updated experience with 306 patients. *World J. Urol.* 1996; **14**: 22–6.
5 Studer UE, Zingg EJ. Ileal orthotopic bladder substitute. What we have learned from 12 years' experience with 200 patients. *Urol. Clin. North Am.* 1997; **24**: 781–93.
6 Arai Y, Taki Y, Kawase N *et al.* Orthotopic ileal neobladder in male patients: functional outcomes of 66 cases. *Int. J. Urol.* 1999; **6**: 388–92.
7 Arai Y, Okubo K, Konami T *et al.* Voiding function of orthotopic ileal neobladder in women. *Urology* 1999; **54**: 44–9.
8 Madersbacher S, Mohrle K, Burkhard F, Studer UE. Long-term voiding pattern of patients with ileal orthotopic bladder substitutes. *J. Urol.* 2002; **167**: 2052–7.
9 Steers WD. Voiding dysfunction in the orthotopic neobladder. *World. J. Urol.* 2000; **18**: 330–7.
10 Hautmann RE. Urinary diversion: ileal conduit to neobladder. *J. Urol.* 2003; **169**: 834–42.
11 Parekh DJ, Gilbert WB, Smith JA Jr. Functional lower urinary tract voiding outcomes after cystectomy and orthotopic neobladder. *J. Urol.* 2000; **163**: 56–8; discussion 58–9.
12 Carrion R, Arap S, Corcione G. *et al.* Confederation of American Urology. A multi-institutional study of orthotopic neobladders: functional results in men and women. *BJU Int.* 2004; **93**: 803–6.
13 Stein JP, Dunn MD, Quek ML, Miranda G, Skinner DG. The orthotopic T pouch ileal neobladder: experience with 209 patients. *J. Urol.* 2004; **172**: 584–7.
14 Furukawa T, Hattori R, Kinukawa T *et al.* [Long-term results and quality of life of hemi-Kock orthotopic ileal neobladder after radical cystectomy.] *Acta Urol. Jpn* 1998; **44**: 461–7. (In Japanese.)
15 Mikuma N, Hirose T, Yokoo A, Tsukamoto T. Voiding dysfunction in ileal neobladder. *J. Urol.* 1997; **158**: 1365–8.
16 Koraitim MM, Atta MA, Foda MK. Desire to void and force of micturition in patients with intestinal neobladder. *J. Urol.* 1996; **155**: 1214–16.
17 Madersbacher S, Schmidt J, Eberle JM *et al.* Long-term outcome of ileal conduit diversion. *J. Urol.* 2003; **169**: 985–90.

International Journal of Urology (2006) **13**, 524–528

Original Article

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

SHINICHI NAKANISHI, HIROYUKI NISHIYAMA, MASAAKI ITO, KOJI YOSHIMURA, TOSHIYUKI KAMOTO AND OSAMU OGAWA

Department of Urology, Kyoto University, Graduate School of Medicine, Kyoto, Japan

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.

Correspondence: Osamu Ogawa MD, Kyoto University, Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.
Email: ogawao@kuhp. kyoto-u.ac.jp

Received 02 May 2005; accepted 20 October 2005.

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.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, and Technology of Japan.

References

1 Jonson DE, Wishnow KI. Are frozen-section examinations of ureteral margins required for all patients undergoing radical cystecomy for bladder cancer? *Urology* 1989; **33**: 451–4.
2 Sharma TC, Melamed MR. Carcinoma in situ of the ureter in patients with bladder carcinoma treated by cystectomy. *Cancer* 1970; **26**: 583–7.
3 Schoenberg MP, Cater HB. Ureteral frozen section analysis during cystectomy: a reassessment. *J. Urol.* 1996; **155**: 1218–20.
4 Zincke H, Garbeff PJ. Upper urinary tract transitional cell cancer after radical cystectomy for bladder cancer. *J. Urol.* 1984; **131**: 50–2.
5 Linker DG, Whitmore WF. Ureteral carcinoma in situ. *J. Urol.* 1975; **113**: 777–80.
6 Union International Contre Cancer. *UICC TNM Classification of Malignant Tumors*, 5th edn. Springer-Verlag, Berlin, 1997.
7 The Japanese Urological Association and the Japanese Society of Pathology. *General Rule for Clinical and Pathological Studies on Bladder Cancer*, 2nd edn. Kanahara-Shuppan, Tokyo, 1993.

8 Culp OS, Utz DC. Experiences with ureteral carcinoma in situ detected during operation for vesical neoplasma. *J. Urol.* 1967; **97**: 679–82.
9 Cooper PH, Waisman J. Severe atypia of transitional epithelium and carcinoma of the urinary bladder. *Cancer* 1973; **31**: 1055–60.
10 Nishio Y, Kaku S. Four cases of upper urinary tract transitional cell carcinoma after radical cystectomy for bladder cancer. *Hinyokika Kiyo* 1988; **34**: 1593–99.
11 Scellhammer PF, Whitmore WF. Transitional cell carcinoma of the urethra in men having cystectomy for bladder cancer. *J. Urol.* 1976; **115**: 56–60.
12 Koss LG, Tiamson EM. Mapping cancerous and precancerous bladder. *JAMA* 1974; **227**: 281–86.

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

Hiroshi Tsuruta,[1,2] Hiroyuki Kishimoto,[2] Takehiko Sasaki,[3] Yasuo Horie,[4] Miyuki Natsui,[2] Yoshiko Shibata,[1] Koichi Hamada,[2] Nobuyuki Yajima,[2] Koichi Kawahara,[2] Masato Sasaki,[2] Norihiko Tsuchiya,[1] Katsuhiko Enomoto,[5] Tak Wah Mak,[6] Toru Nakano,[7] Tomonori Habuchi,[1] and Akira Suzuki[2]

Departments of [1]Urology, [2]Molecular Biology, [3]Microbiology, [4]Gastroenterology, and [5]Pathology, Akita University School of Medicine, Akita, Japan; [6]The Campbell Family Institute for Breast Cancer Research, and Departments of Immunology and Medical Biophysics, University of Toronto, Toronto, Ontario, Canada; and [7]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 [*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

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

T. Habuchi and A. Suzuki contributed equally to this work as last authors.

Requests for reprints: Akira Suzuki, Department of Molecular Biology, Akita University School of Medicine, Hondo 1-1-1, Akita 010-8543, Japan. Phone: 81-18-884-6077; Fax: 81-18-884-6077; E-mail: suzuki@med.akita-u.ac.jp.

doi:10.1158/0008-5472.CAN-05-4627

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′-CTCCCACCAATGAA-CAAACAGT-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′-CTCCCACCAATGAA-CAAACAGT-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.

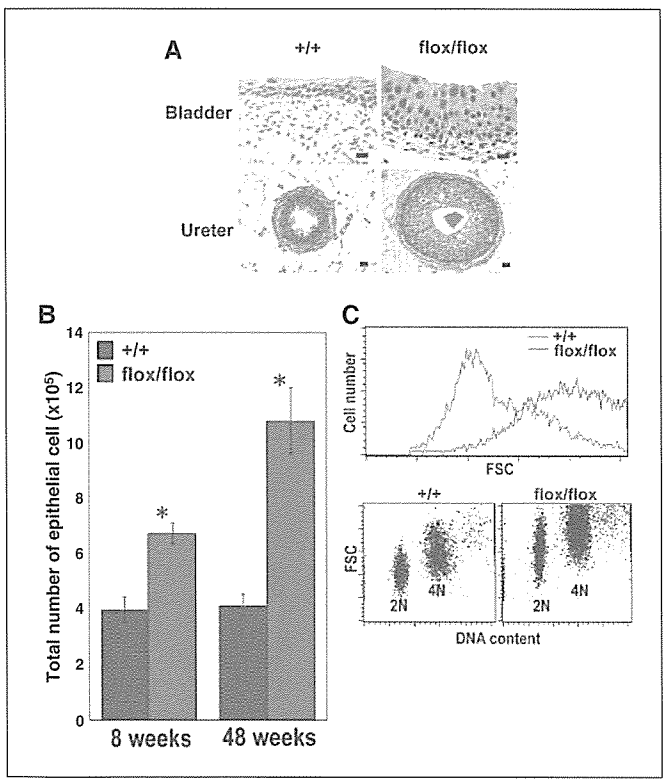

Figure 1. Urothelial hyperplasia in *FPten*$^{flox/flox}$ mice. *A*, H&E staining of mouse bladder sections. *Left*, normal bladder and ureter epithelial cells from an 8-week-old WT (+/+) mouse; *right*, urothelial hyperplasia of the bladder and ureter from an 8-week-old *FPten*$^{flox/flox}$ (*flox/flox*)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

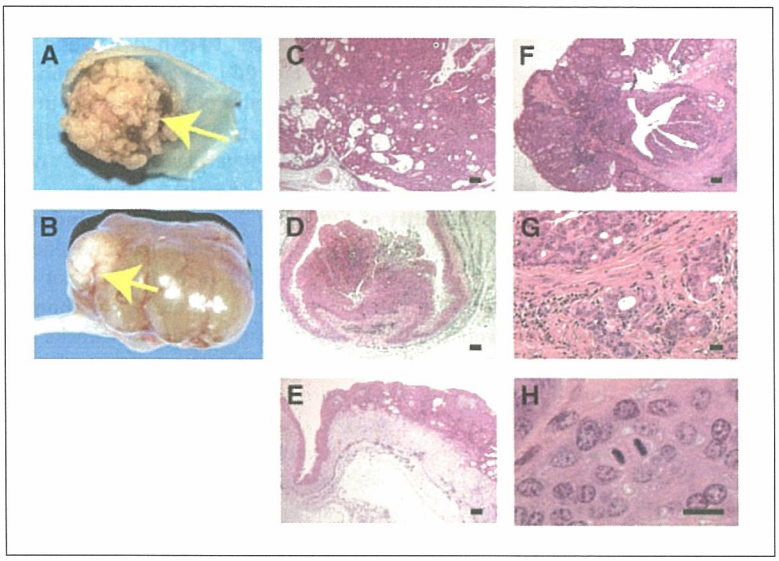

Figure 2. Spontaneous superficial papillary carcinomas in *FPten*$^{flox/flox}$ mice. Gross and histologic analyses of urothelial cancers in *FPten*$^{flox/flox}$ mice. *A* and *B,* gross appearance of a pedicellate papillary bladder carcinoma (*A; arrow*) and a pelvic carcinoma (*B; arrow*) exhibiting 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

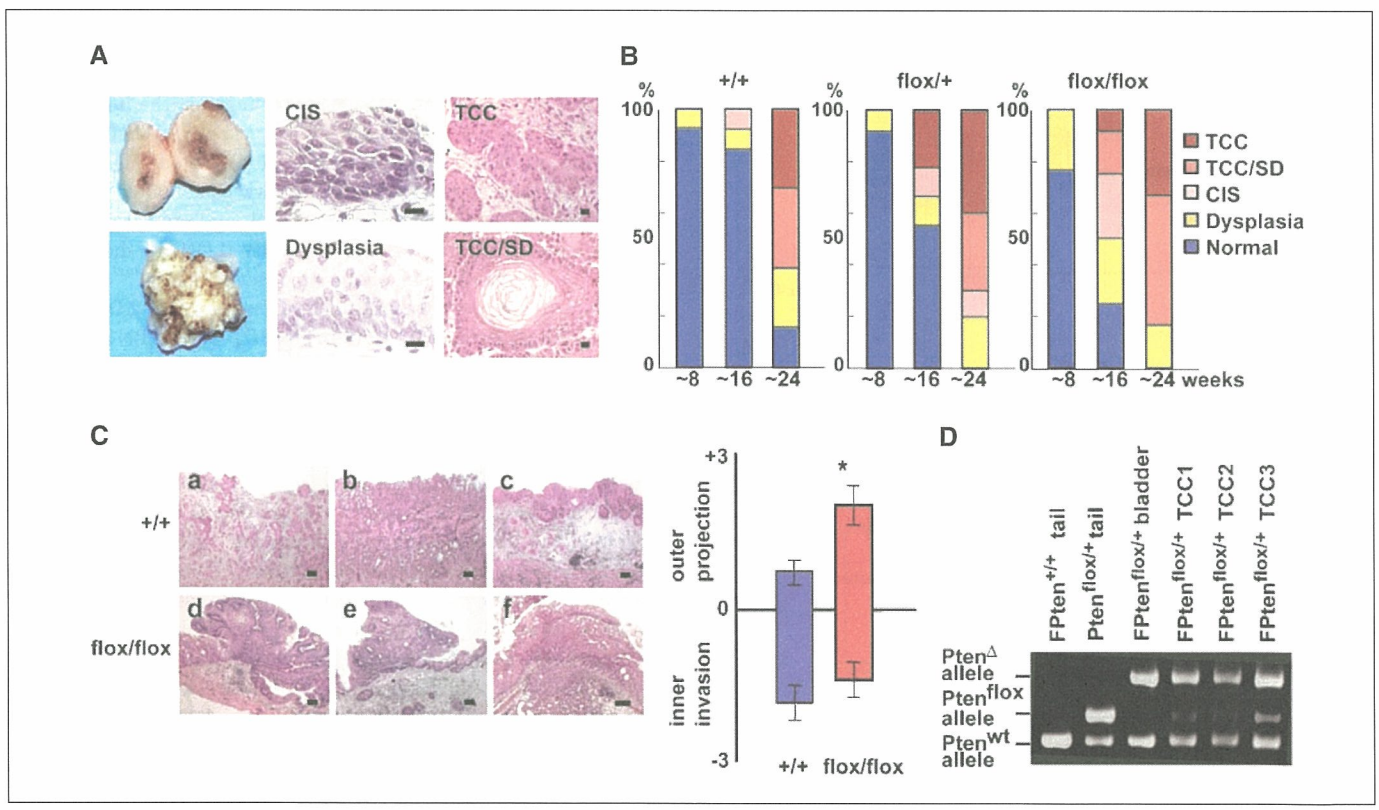

Figure 3. Acceleration of BBN-induced urothelial tumorigenesis. *A,* representative tumors observed following BBN administration. *Left,* gross appearance of tumors observed in WT (*top*) and *FPten*$^{flox/flox}$ (*bottom*) mice. *Middle* and *right,* dysplasia, CIS, TCC, and TCC with squamous cell 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).

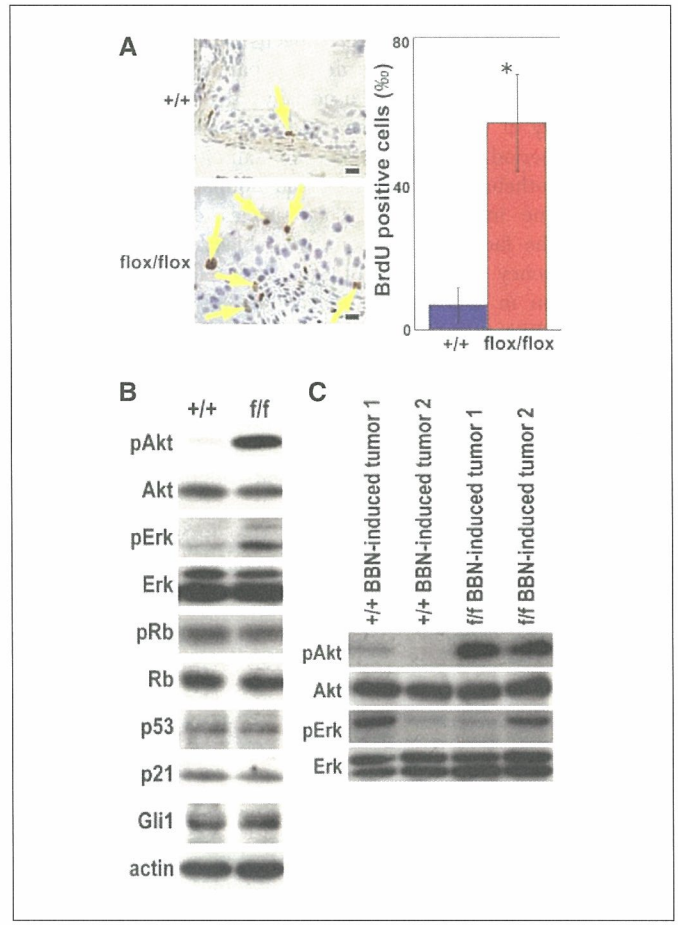

Figure 4. Increased bladder epithelial cell proliferation and enhanced phosphorylation of Akt and ERK in *FPten*$^{flox/flox}$ mice. *A,* increased bladder epithelial cell proliferation. *Left,* BrdUrd$^+$ bladder epithelial cells (*arrows*) from 10-week-old WT and *FPten*$^{flox/flox}$ mice were counted 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.

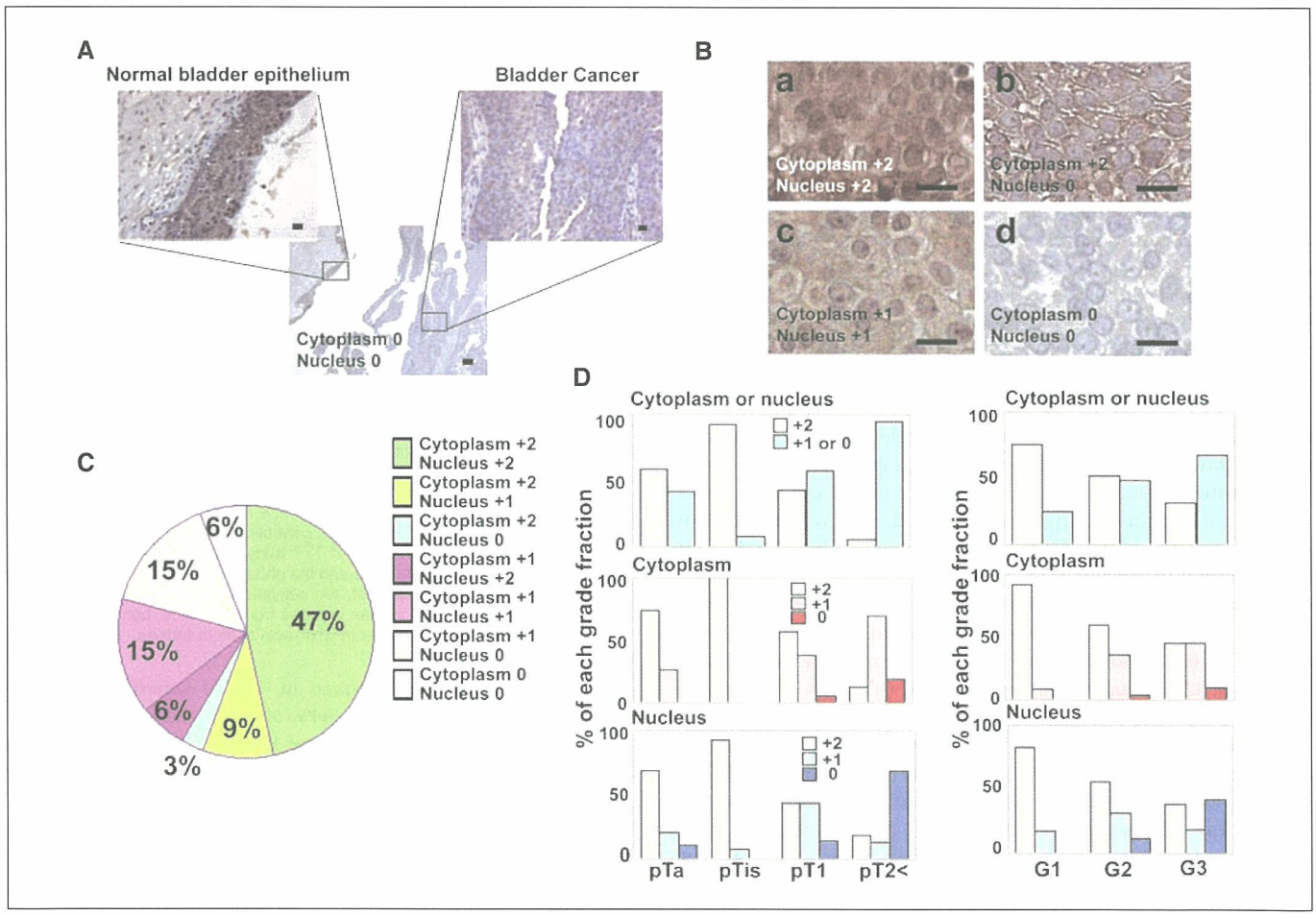

Figure 5. Reduction or absence of PTEN expression in human primary bladder cancers. *A* and *B,* representative urothelial sections immunostained for PTEN protein expression. *A, left,* region of normal bladder epithelium (score +2); *right,* adjacent region showing the epithelium of a primary bladder 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).