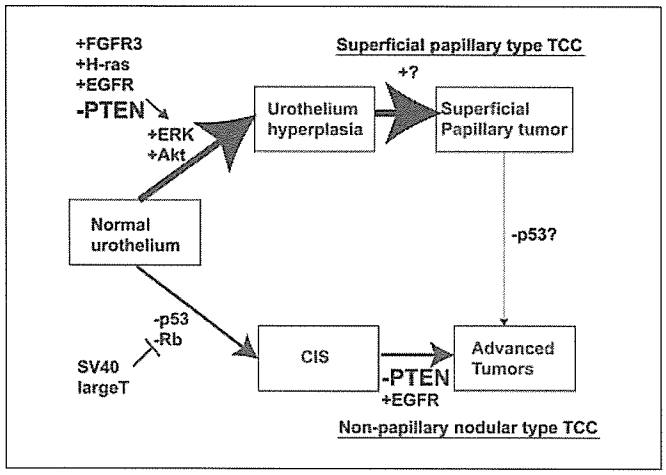

Figure 6. Model of bladder cancer progression. *Top pathway,* overexpression of FGFR3, EGFR, or H-Ras, coupled with loss of PTEN, promotes cell proliferation by activating the Akt and ERK pathways. Urothelial hyperplasia is induced that can progress to superficial papillary TCC following additional (unknown)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

observed in *FPten*$^{flox/flox}$ mice for only 1 week after birth due to the induction of p21. In contrast, we observed increased bladder epithelial cell proliferation even in 10-week-old *FPten*$^{flox/flox}$ mice. Moreover, the total number of bladder epithelial cells was much higher in our *FPten*$^{flox/flox}$ mice at 48 weeks than at 8 weeks of age, and p21 was not induced. The loss of Akt activation coupled with p21 induction in *FPten*$^{flox/flox}$ bladder epithelial cells as observed by Yoo et al. could have occurred as the epithelial cells were peeled off the bladder wall. Alternatively, the cells may have been damaged during a cell separation procedure.

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

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

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

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

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

Acknowledgments

Received 12/28/2005; revised 5/5/2006; accepted 6/23/2006.

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

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

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

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Accepted for publication 5 June 2006

OBJECTIVE

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

PATIENTS AND METHODS

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

RESULTS

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

CONCLUSION

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

KEYWORDS

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

INTRODUCTION

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

PATIENTS AND METHODS

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

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

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

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

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

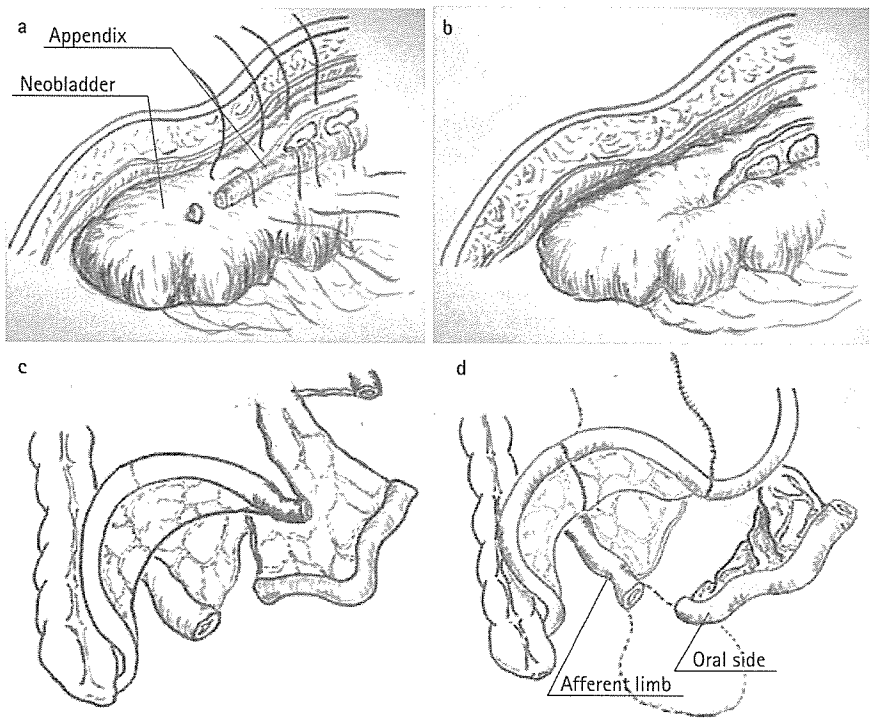

TABLE 1 Patients with urethral recurrence after an ileal neobladder

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

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

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

RESULTS

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

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

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

DISCUSSION

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

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

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

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

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

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

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

CONFLICT OF INTEREST

None declared.

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Abbreviations: **TUR**, transurethral resection.

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Jpn J Clin Oncol 2006;36(2)116–120
doi:10.1093/jjco/hyi225

Cancer Genetics Report

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

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Received September 13, 2005; accepted November 30, 2005; published online January 17, 2006

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

Key words: PIG3 – bladder cancer – VNTRs

INTRODUCTION

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

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

SUBJECTS AND METHODS

SUBJECTS

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

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used, although not all TCC patients treated during study periods were included because materials or informed consents were not available. Clinical and histopathological information was obtained from patient charts and pathological reports. The cancer stage and grade were assigned according to the tumor-node-metastasis (TNM) staging system (15) and the World Health Organization (WHO) criteria (16,17).

A total of 338 native Japanese people in Akita Prefecture, who visited community hospitals for a routine health checkup, were recruited for the study as controls. All control subjects were checked by routine urinalysis with microscopic examination of the urine sediment, urinary cytology, prostate specific antigen and ultrasonography to rule out the presence of urinary tract cancers. Except for clear evidence of urinary tract cancers, no exclusion criteria were provided for the recruitment of controls.

The group of TCC patients comprised of 207 males and 66 females and the group of controls comprised of 269 males and 69 females. The mean age ± SD was 61.2 ± 12.7 and 66.3 ± 11.6 years, respectively. The gender distributions and the mean ages were not statistically different between both groups. This study was approved by the Institutional Review Board of the Kyoto University Graduate School and the Akita University School of Medicine. Written informed consent to participate in the study was obtained from each patient before surgery, according to the ethical guidelines.

Genotyping of the *PIG3* Promoter VNTRs

DNAs were extracted from blood samples collected from subjects using a QIAamp Blood Kit (QIAGEN, Hilden, Germany) or by the standard method with proteinase K digestion followed by phenol/chloroform extraction. The fragment encompassing the *PIG3* promoter region was amplified using the specific forward primer 5′-TGCGGTGCCAGCCTGAGGCT-3′ and fluorescent dye 6-FAM labeled reverse primer 5′-TTCCGGTCCTCCCGGCTTGT-3′. PCRs were carried out in a 25 μl volume containing 20 ng of genomic DNA, a commercial 1× PCR buffer, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1.5 mM $MgCl_2$, 50 pmol of each primer and 1.0 U of Ampli-Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ). After a 10 min initial denaturation step at 95°C, 35 cycles of PCR consisting of 95°C for 30 s, 68°C for 30 s and 72°C for 60 s were carried out, followed by a 7 min final extension step at 72°C in a thermal cycler (TaKaRa PCR Thermal Cycler MP: TaKaRa BIOMEDICALS, Kusatsu, Japan). After confirmation of successful PCR amplification with 2.0% agarose gel electrophoresis, PCR products were run on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems-Roche, Branchburg, NJ), and allele sizes were assigned using the GeneScan software (PE Applied Biosystems-Roche). The number of (TGYCC)*n* repeats was calculated from the size of the PCR products in relation to a series of standards and confirmed by the direct sequencing of PCR products in representative cases or 14% PAGE and silver nitrate staining.

TP53 Immunohistochemistry

The status of the p53 protein in cancer tissue was assessed with immunohistochemistry using an anti-p53 antibody (18). In brief, after the deparaffinization and blocking of endogenous peroxidase with hydroxyl peroxide, antigen retrieval was performed using a microwave oven. The anti-p53 mouse monoclonal antibody Do-7 (Novocastra, Newcastle-upon-Tyne, UK) was incubated overnight at a dilution of 1:100 with specimens at 4°C. Staining was achieved using the Dako LSAB kit (Dako, Carpinteria, CA). Tumors with >10% immunoreactivity in nuclei were judged as positive stainings and defined as TP53 mutant-type cancers, and otherwise defined as TP53 wild-type cancers. Two investigators (J.W. and H.K.) independently assessed the results of immunostaining. When discordant diagnosis were judged by investigators, results were reevaluated and discussed until the agreement was reached.

Statistical Analysis

All data were entered into an access database and analyzed with Stat-View (version 5.0) software. In analyzing the relationship between the genotype and disease status of TCC, the genotype frequencies of each category of cancer grade and stage were compared against those of the controls. Data were analyzed with 2×2 contingency tables according to the genotype using the chi-square test. A multivariate logistic regression model was used to assess the relative risk [age and gender adjusted odds ratio (aOR) and 95% confidence interval (95% CI)] for bladder TCC risk and higher disease status. The frequencies of the PIG3 promoter VNTRs genotype between the TP53 status were compared using the chi-square test. All statistical results were considered significant if the *P*-value was <0.05.

RESULTS

The *PIG3* Promoter VNTRs Genotypes and the Risk of the Bladder TCC

Genotyping using PCR methods demonstrated that there were seven VNTRs in the *PIG3* promoter; which were 10, 12, 14, 15, 16, 17 and 18 repeats of the pentanucleotide sequences (TGYCC) (Fig. 1). As for the allele frequency, there was no

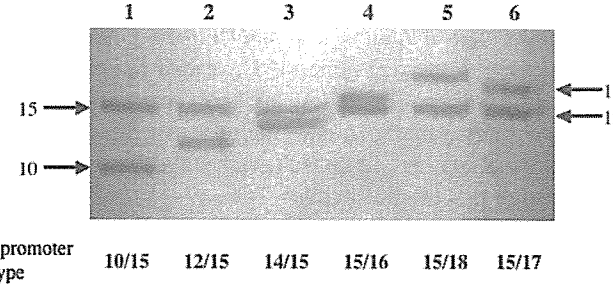

Figure 1. Genotyping of the *PIG3* promoter VNTRs. Lanes 1, 2, 3, 4, 5 and 6 represent 10/15, 12/15, 14/15, 15/16, 15/18 and 15/17 times repeats genotypes, respectively. Arrows with numbers denote the number of times of repeat.

Table 1. Characteristics of subjects and the *PIG3* promoter VNTRs subtypes

	Number (%) of *PIG3* promoter subtype			Chi-square *P*-value	Univariate	
	Total	Short repeat subtype[1]	Long repeat subtype[2]		Adjusted OR[3] (95% CI)	*P*-value
Controls	338	42 (12.4)	296 (87.6)		1.00 (reference)	
TTC[4] patients	273	39 (14.3)	234 (85.7)	0.500	0.89 (0.54–1.48)	0.653
Grade						
Low[5]	175	19 (10.9)	156 (89.1)	0.602	0.77 (0.46–1.45)	0.417
High[6]	98	20 (20.4)	78 (79.6)	0.038	1.71 (0.95–3.18)	0.093
Stage						
≤pT1	218	28 (12.8)	190 (87.2)	0.885	0.90 (0.51–1.56)	0.699
≥pT2	55	11 (20.0)	44 (80.0)	0.146	2.31 (1.05–5.90)	0.038

All *P*-values are against controls.
[1]Short repeat subtype means the patients with at least one allele of 14 or shorter repeat.
[2]Long repeat subtype means the other patients.
[3]TCC: transitional cell carcinoma.
[4]Low grade = Grade 1 + 2.
[5]High grade = Grade 3.
[6]Odds ratios are adjusted for age and gender.

significant difference between the control group (10 = 6.7%, 12 = 0.2%, 14 = 0.4%, 15 = 88.6%, 16 = 2.8%, 17 =1.3% and 18 = 0%) and the TCC group (10 = 7.2%, 12 = 0%, 14 = 0.2%, 15 = 84.5%, 16 = 5.1%, 17 = 2.8% and 18 = 0.2%, $P = 0.09$). The genotype distribution of both groups was in the Hardy–Weinberg equilibrium ($P = 0.94$ in the control group and $P = 0.72$ in the TCC group). As the allele of 15 repeats was the most frequent and considered to be the standard one, the *PIG3* promoter VNTRs genotypes were categorized into two groups; a short repeat subtype, which was defined as subjects who harbored at least one allele of 14 or less repeats of the VNTR (10/10, 10/15–17, 12/15 and 14/15) and a long repeat subtype, which included the other subjects. The distributions of the *PIG3* promoter subtype were not significantly different between both genders. There was also no significant difference in the frequencies of the short repeat subtypes between the controls and the patients (12.4 and 14.3%, respectively, $P = 0.500$; Table 1).

The Relationship of the *PIG3* Promoter VNTRs and Cancer Grade/Stage

To evaluate whether the *PIG3* promoter VNTRs were associated with an advanced disease status of the bladder TCCs, the frequencies of the short repeat subtype were compared as for their cancer grades and stages. In terms of the cancer grade, the frequency of the short repeat subtype was significantly higher in the high grade (Grade 3) TCC patients than in the controls (20.4 and 12.4%, $P = 0.038$), whereas there were no significant differences in the frequencies of the short repeat subtype between the low grade (Grade 1–2) TCC patients and the controls (10.9 and 12.4%, $P = 0.602$; Table 1). As for the cancer stage, logistic regression analysis demonstrated that the subjects with the short repeat subtype had a significantly higher risk for high stage (≥pT2) cancers compared with the controls (aOR = 2.31, 95% CI = 1.05–5.90, $P = 0.038$; Table 1). No significantly higher risk of low stage (≤pT1) TCC against the controls was demonstrated (aOR = 0.90, 95% CI = 0.51–1.56, $P = 0.699$; Table 1).

Table 2. Association of TP53 status and the *PIG3* promoter VNTRs subtypes

	Number (%) of *PIG3* promoter subtype			
	Total	Short repeat subtype	Long repeat subtype	Chi-square *P*-value
TP53 wild-type	23	5 (21.7%)	18 (78.3%)	
TP53 mutant-type[1]	22	0 (0%)	22 (100%)	0.020

[1]TP53 mutant-type was defined as cancers with >10% immunoreactivity in nuclei against Do-7 anti-p53 Ab.

The Status of Tp53 and the *PIG3* Promoter VNTRs Subtypes

We investigated the association of the TP53 status of cancer tissues and *PIG3* promoter subtype. Among the 51 patients who were treated with radical cystectomy for invasive TCCs, 45 surgical specimens of the same number of patients were available for immunohistochemistry of TP53. Twenty-three cancers were judged as negative staining and defined as TP53 wild-type, and the other 22 cancers were defined as TP53 mutant-type. Among the 23 patients with TP53 wild-type cancers, 5 (21.7%) and 18 (78.3%) were classified into the short and the long repeat subtypes of the *PIG3* promoter, respectively. On the other hand, all 22 patients with TP53 mutant-type cancers were of the long repeat subtype. The frequency of the short repeat subtype was statistically higher in the TP53 wild-type invasive TCCs than that in the TP53 mutant-type ones ($P = 0.020$; Table 2).

DISCUSSION

In the present study, the *PIG3* promoter VNTRs were analyzed in Japanese patients with bladder TCCs and healthy controls. In the healthy controls, the 15 repeats allele was dominant (88.6%), and the allele frequency for the 14 or less repeats was 7.3%. There were newly identified alleles of 12, 14 and 18 times repeats, which have not been reported in the German and Greek populations (14,19). The discordant result may come from racial differences.

The allele frequency of the 14 or less repeats in our series was not significantly different from those in the previous report from Germany (5.1%, $P = 0.264$ by chi-square test) (14). As for the significance of the *PIG3* promoter VNTRs in the susceptibility to cancers, Gorgoulis et al. (19) reported that the frequencies of the VNTRs in breast and lung cancer patients were not significantly different from those of healthy controls, which was consistent with our results of those with bladder TCCs. However, when the short repeat subtype was defined as the patients with at least one allele of 14 or less repeats, the prevalence of the short repeat subtype was significantly higher in the high grade TCC patients than in the controls and was associated with a higher risk of advanced stage. The frequency of short repeat subtype of the *PIG3* promoter VNTRs was not significantly different between the controls and high stage TCCs with chi-square test, but it was a significant risk factor of high stage TCCs with logistic regression analysis, which suggests that the advanced stage was also associated with the *PIG3* promoter VNTRs. On the other hand, when the short repeat subtype was defined as those with at least one allele of 15 or less repeats, no significant difference was found between the normal and high-grade/stage TCCs (data not shown). Although there has been no report that analyzed the relation of the *PIG3* promoter VNTRs to its expression level and the cellular phenotype, these data indicated that the existence of at least one allele of 14 or less repeats was associated with the susceptibility of high grade/high stage TCCs. In combination with the evidence that shorter repeats of VNTRs was related to lower expression levels of *PIG3*, the existence of at least one allele of 14 or less repeats might have resulted in the haploinsufficiency of *PIG3* expression.

In the pathogenesis of bladder TCC, two different pathways have been proposed; the superficial TCC pathway and the invasive TCC pathway (9,20). Alterations of TP53 may play key roles in the development of invasive TCC, as the mutations in *p53* are identified in over half of them, in contrast to superficial papillary TCC. Furthermore, the mutations in *p53* may affect the sensitivity to chemotherapy and the prognosis (20). It was demonstrated in an *in vitro* study that the *PIG3* promoter VNTRs is directly bound by TP53, and that the activity of *PIG3* was influenced according to the number of repeats within it (14). Interestingly, our immunohistochemical analysis for TP53 indicated that the frequency of the *PIG3* short repeat subtype was statistically higher in the patients with TP53 wild-type TCCs than those with TP53 mutant-type TCCs. TP53 seems to bind to the promoter of *PIG3*, and PIG3 was involved in TP53 mediated apoptosis. Mutations of TP53 or the presence of the short repeat of the promoter might down regulate the activation of PIG3, resulting in inhibition of apoptosis and induction of malignant phenotype. Although the sample number is too small ($N = 45$), these data suggested that the suppression of the TP53–PIG3 pathways might be critical for the tumorigenesis of high grade/high stage bladder TCC.

Originally, *PIG3* was identified as one of the genes upregulated in the course of the TP53 induced, reactive oxygen species (ROS) mediated apoptosis (13,21,22) and has a homology with NAD (P) H quinone oxidoreductase-1 (*NQO1*), although its exact biological function has not been well elucidated. NQO1 suppresses DNA damage due to ROS by preventing the one-electron reduction of quinones by cytochrome P450 (23) and generates antioxidant forms of ubiquinone (24) and α-tocopherol (25). Interestingly, polymorphisms of *NQO1* have also been reported to be associated with the tumorigenesis of bladder cancer (26). Thus, PIG3 might play roles in regulating ROS and be associated with the invasive bladder TCC, like as NQO1. It would be interesting to analyze the function of PIG3 in the cancer cells.

Our preliminary results as for the association of the *PIG3* promoter VNTRs with the progression of bladder cancer was marginally significant, but the result was limited due to relatively small number of ≥T2 cases. Therefore, larger-scale study, that puts emphasis on the patients with ≥T2 bladder cancer, would be needed to verify our results.

Acknowledgements

This work was supported by Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports and Technology of Japan and by Suzuki Foundation for Urological Medicine.

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