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

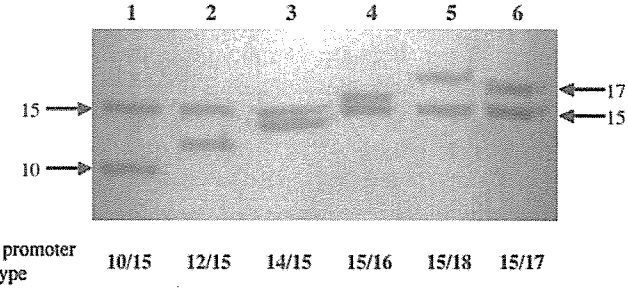

Figure 1. Genotyping of the *PIG3* promoter VNTRs. Lanes 1, 2, 3, 4, 5 and 6 represent 10/15, 12/15, 14/15, 15/16, 15/18 and 15/17 times repeats genotypes, respectively. Arrows with numbers denote the number of times of repeat.

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

	Number (%) of *PIG3* promoter subtype			Chi-square	Univariate	
	Total	Short repeat subtype[1]	Long repeat subtype[2]	*P*-value	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.

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References

1. Foresman WH and Messing, EM. Bladder Cancer: natural History, tumor markers, and early detection strategies. *Semin Surg Oncol* 1997;13:299–306.
2. Kinoshita H, Ogawa O, Kakehi Y, Mishina M, Mitumori K, Itoh N, et al. Detection of telomerase activity in exfoliated cells in urine from patients with bladder cancer. *J Natl Cancer Inst* 1997;89:724–30.
3. Miyanaga N, Ohtani M, Akaza H, Koiso K. Clinical statistical studies on bladder cancer in Japan. *Nihon Rinsyo* 1993;51:823–9.
4. Takagi Y, Takashi M, Koshikawa T, Ohshima S. Immunohistochemical demonstration of cyclin D1 in bladder cancers as an inverse indicator of invasiveness but not an independent prognostic factor. *Int J Urol* 2000;7:366–72.
5. Wagner U, Suess K, Luginbuhl T, Schmid U, Ackermann D, Zellwegener T. Cyclin D1 overexpression lacks prognostic significance in superficial urinary bladder cancer. *J Pathol* 1999;188:44–50.
6. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991;253:49–53.
7. Cordon-Cardo C, Dalbagani G, Saez GT, Oliva MR, Zhang ZF, Rosai J, et al. p53 mutations in human bladder cancer: genotypic versus phenotypic patterns. *Int J Cancer* 1994;56:347–53.
8. Sarkis AS, Dalbagani G, Cordon-Cardo C, Melamed J, Zhang ZF, Sheinfeld J, et al. Association of p53 nuclear overexpression and tumor progression in carcinoma in situ of the bladder. *J Urol* 1994;152:388–92.

9. Spruck CH, Ohnesit PF, Gonzares-Zulueta M, Esrig D, Miyao N, Tsai YC, et al. Two molecular pathways to transitional cell carcinoma of the bladder. *Cancer Res* 1994;54:784–8.
10. Esrig D, Spruck CH, Nicols PW, Chaiwun B, Steven K, Groshen S, et al. p53 nuclear protein accumulation correlates with mutations in the p53 gene, tumor grade, and stage in bladder cancer. *Am J Pathol* 1993;143:1389–97.
11. Habuchi T, Takahashi R, Yamada H, Ogawa O, Kakehi Y, Ogura S, et al. Influence of cigarette smoking and schistosomiasis on p53 gene mutation in urothelial cancer. *Cancer Res* 1993;53:3795–9.
12. el-Deiry MS, Harper JW, O'Conner PM, Velculescu VE, Canman CE, Jackman J, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994;54:1169–74.
13. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. *Nature* 1997;389:300–5.
14. Contente A, Dittmer A, Koch MC, Roth J, Dobbelstein M. A polymorphic microsatellite that mediates induction of PIG3 by p53. *Nat Genet* 2002;30:315–20.
15. Fleming ID, Cooper JS, Henson DE, Hutter RVP, Kennedy BJ, Murphy GP, et al. AJCC Cancer Staging Manual. 5th edn. Philadelphia: Lippincott-Raven 1997; 241–3.
16. The Japanese Urological Association and the Japanese Society of Pathology. General Rule for Clinical and Pathological Studies on Bladder Cancer. 3rd edn. Tokyo: Kanahara-Shuppan 2001; 56–8.
17. Mostofi FK, Sobin L, Torloni H. Histological typing of urinary bladder tumors. In: International Histological Classification of Tumors, No. 10. Geneva: World Health Organization 1973; 15–34.
18. Watanabe J, Nishiyama H, Okubo K, Takahashi T, Toda Y, Habuchi T, et al. Clinical evaluation of p53 mutations in urothelial carcinoma by IHC and FASAY. *Urology* 2004;63:989–93.
19. Gorgoulis VG, Liloglou T, Sigala F, Korkolis D, Yannoukakos D, Papalambros E, et al. Absence of association with cancer risk and low frequency of alterations at a p53 responsive *PIG3* gene polymorphism in breast and lung carcinomas. *Mut Res* 2004;556:43–50.
20. Catherine AR, Somdatta S, Knut PJ, Melissa SB, Jairaj AP, David FJ. Genetic alterations and biological pathways in human bladder cancer pathogenesis. *Urol Oncol* 2000;5:191–203.
21. Flatt PM, Polyak K, Tang LJ, Scatena CD, Westfall MD, Rubinstein JY, et al. p53 -dependent expression of *PIG3* during proliferation, genotoxic stress, and reversible growth arrest. *Cancer Lett* 2000;156:63–72.
22. Venot C, Maratrat M, Dureutil C, Conseiller E, Bracco L, Debussche L. The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. *EMBO J* 1998;17:4668–79.
23. Chesis PL, Levin DE, Smith MT, Lars E, Ames BN. Mutagenicity of quinones: Pathways of metabolic activation and detoxification. *Proc Natl Acad Sci USA* 1984;81:1696–700.
24. Beyer RE, Segura-Aguliar J, Di Bernardo M, Cavazzoni R, Fato R, Fiorentini D, et al. The role of DT- diaphorase in the maintenance of the reduced antioxidant form of coenzyme Q in membrane systems. *Proc Natl Acad Sci USA* 1996;93:2528–32.
25. Siegel D, Bolton EM, Burr JA, Liebler DC, Ross D. The reduction of α-tocopherolquinone by human NAD(P)H:quinone oxidoreductase: The role of α-tocopherolhydroquinone as a cellular antioxidant. *Mol Pharmacol* 1997;52:300–5.
26. Schulz WA, Kummeck A, Rosinger I, Eickelmann P, Neuhas C, Ebert T, et al. Increased frequency of a null allele for NAD(P)H: quinone oxidoreductase in patients with urological malignancies. *Pharmacogenetics* 1997;7:235–9.