Table 3 Univariate and multivariate analysis of factors influencing recurrence

Variables	Hazard ratio	95% Confidential interval	*P*-value
Univariate analysis			
Group (observation/maintenance)	5.7503	0.7073–46.7518	0.0467
Age (70 years or older/younger than 70 years)	0.4951	0.1183–2.0719	0.3264
Smoking habit (yes/no)	4.1639	0.5115–33.9003	0.1159
Stage (CIS/Ta,T1)	0.6193	0.0759–5.0532	0.6734
Tumor status (primary/recurrent)	0.3291	0.0405–2.6770	0.2354
Multivariate analysis			
Group (observation/maintenance)			
Adjusted for smoking habit	5.1078	0.6254–41.7154	0.0676

CIS, carcinoma *in situ*.

Table 4 Adverse drug reactions during the induction therapy and adverse drug reactions in the maintenance group

Adverse event	Frequency (%)	Grade		
		Grade 1	Grade 2	Grade 3
Induction therapy				
Pain on urination	82.2	52.2	25.6	4.4
Urinary frequency	82.2	47.8	27.8	6.7
Gross hematuria	72.2	62.2	8.9	1.1
Difficulty with urination	52.2	46.7	4.4	1.1
Fever (38°C or higher)	30.0	27.8	2.2	0.0
Arthritis/arthralgia	5.6	1.1	3.3	1.1
Muscle pain	1.1	0.0	0.0	1.1
ALT elevation	7.8	4.4	2.2	1.1
AST elevation	7.8	6.7	0.0	1.1
gamma-GTP elevation	12.2	11.1	0.0	1.1
Maintenance group				
Urinary frequency	20.8	8.3	4.2	8.3
Pain on urination	16.7	8.3	4.2	4.2

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

in the observation group died of bladder cancer. Two-year overall survival rate was 91.7% in the maintenance group and 92.6% in the observation group, without a significant difference between the groups ($P = 0.885$). When the independent contribution of each background factor to recurrence was examined using univariate analysis, maintenance therapy was identified as a significant predictor (Table 3), but when multivariate analysis was used, it was not an individually significant factor influencing recurrence ($P = 0.0676$).

Safety was evaluated in 90 patients. Table 4 shows the ADR with frequency ≥10% or grade ≥3 during the induction therapy. Although urination-related local symptoms occurred in 82.2% of patients, grade 3 urinary events had low frequencies. The frequencies of ADR not related to urination were low, except for pyrexia (38°C or higher), with an incidence rate of 30.0%. These ADR resolved with/without anti-inflammatory agents or corticosteroid preparations. Table 4 shows ADR with a frequency ≥10% or grade ≥3 in the maintenance group. These ADR also resolved with/without anti-inflammatory agents. The frequency of ADR during maintenance therapy was lower than that during induction therapy.

According to EORTC QLQ-C30, higher scores on the functioning scales and lower scores on the symptom scales indicate a better QOL. There were no significant differences in the QOL scores during induction therapy for all categories (data not shown). QOL on the emotional functioning scale was improved significantly ($P = 0.004$, data not shown). Most symptom scores tended to improve, although

Table 5 Quality of life scores of the maintenance and the observation groups

Scale	Maintenance group			Observation group		
	After induction therapy	14 months after randomization	*P*-value	After induction therapy	14 months after randomization	*P*-value
Physical functioning scale	90.0 (14.7)	95.8 (6.4)	0.145	89.5 (16.6)	89.8 (18.5)	0.945
Role functioning scale	89.4 (22.7)	96.9 (9.1)	0.222	88.5 (14.0)	92.1 (22.7)	0.508
Emotional functioning scale	89.4 (11.8)	88.0 (12.9)	0.736	88.1 (11.1)	85.3 (15.8)	0.476
Cognitive functioning scale	84.9 (17.0)	84.4 (16.6)	0.932	80.1 (14.2)	76.2 (19.4)	0.426
Social functioning scale	88.6 (22.7)	92.7 (17.2)	0.55	89.7 (15.0)	91.3 (22.7)	0.784
Global health status	68.2 (22.1)	77.6 (17.4)	0.165	66.0 (17.6)	63.9 (21.5)	0.709
Fatigue symptom scale	17.7 (17.0)	16.7 (15.2)	0.851	19.2 (14.9)	20.6 (21.5)	0.793
Nausea and vomiting scale	0.0 (0.0)	1.0 (4.2)	0.246	0.0 (0.0)	0.0 (0.0)	—
Pain symptom scale	12.9 (23.0)	5.2 (10.0)	0.22	18.0 (25.4)	8.7 (22.7)	0.201
Dyspnoea symptom scale	9.1 (15.2)	10.4 (16.0)	0.796	9.0 (17.8)	7.9 (14.6)	0.83
Insomnia symptom scale	13.6 (24.5)	12.5 (16.7)	0.873	16.8 (19.4)	9.5 (15.4)	0.177
Appetite loss symptom scale	6.1 (13.2)	2.1 (8.3)	0.295	3.9 (10.9)	4.8 (12.0)	0.785
Constipation symptom scale	16.7 (30.4)	14.6 (27.1)	0.829	18.0 (27.1)	7.9 (14.6)	0.134
Diarrhoea symptom scale	4.6 (11.7)	4.2 (11.4)	0.921	2.6 (9.1)	1.6 (7.3)	0.691
Financial difficulties symptom scale	13.6 (16.8)	10.4 (16.0)	0.555	6.4 (13.4)	6.4 (17.1)	0.989

Values expressed as mean (SD). *P*-value, after induction therapy *vs* 14 months after randomization.

significant differences were not found. QOL after the induction therapy was also compared with that at 14 months after randomization in each group (Table 5). In the maintenance group, none of the functioning scales showed a reduction in QOL after the maintenance therapy compared with before. All the symptom scales, except the nausea and vomiting scale and the dyspnoea symptom scale, indicated an improvement in QOL after BCG treatment compared with before, although none of the improvements showed a significant difference. In the observation group, a significant difference in QOL after randomization compared with that before randomization was not observed.

Discussion

The present study showed a CR rate of 77.0% for CIS of the bladder and 60.0% for Ta and T1 bladder cancer, resulting in an overall CR rate of 75.0%. This result was similar to that of a previous Japanese report.[10] Although BCG intravesical instillation is often associated with undesirable adverse reactions, it was reported to be a highly safe treatment causing few serious ADR.[11] A similar ADR profile was seen in the present study. The frequency of grade ≥3 ADR was low and there were no serious ADR resulting in death.

In the present study, only patients who had achieved CR with the induction therapy were randomly assigned to the maintenance or the observation group. This design allowed the advance exclusion of those who failed to achieve CR with induction therapy and the even assignment to the two groups, as shown in Table 2. In contrast, the design requiring reacquisition of consent before randomization might constitute a selection bias, because not all patients who have achieved CR will provide consent for participation again. Concerning the study design of clinical trials of BCG maintenance instillation, further investigation might be needed.

Although recurrence was confirmed in one patient in the maintenance group and seven patients in the observation group, there was no significant difference in recurrence-free survival between the groups. Univariate analysis showed that maintenance therapy reduced recurrence significantly, but multivariate analysis did not show a significant difference. This might be a result of the small sample size and the existence of various confounding factors.

According to the results of the SWOG randomized clinical trial,[6] a 3-week maintenance schedule seems to be regarded as the standard maintenance instillation method. Decobert *et al.* retrospectively studied the relationship between the number of cycles of 3-week maintenance and recurrence[12] and suggested that a minimum of three maintenance cycles of BCG (nine instillations) was required to significantly improve the no recurrence rate. In the present study, however, the total number of maintenance instillations was set as four instillations, because this study had to be carried out by an approved method for BCG Tokyo strain in Japan. Surprisingly, maintenance consisting of just four instillations decreased the recurrence rate as compared with that in the observation group, and was also identified as a significant factor in preventing recurrence using univariate

analysis, although the present study failed to show significant superiority of the 4-instillation maintenance schedule. The SWOG 8507 trial is the only trial in which efficacy of maintenance therapy had been proved to be significant.[6] This maintenance schedule consists of 81 mg Connaught strain by the 3-week maintenance method at 3- or 6-month intervals, and a total of 21 instillations. Akaza *et al.*[10] carried out a prospective randomized trial using 40 mg Tokyo strain, and 107 patients were randomly assigned to the observation group and the maintenance group which consisted of 40 mg BCG monthly 12 times. There was no significant difference in the 3-year non-recurrence rate. In the present study, the maintenance schedule consisted of 80 mg Tokyo strain at 3-month intervals and a total of four instillations. These findings show that a 3-week maintenance method might be a critical factor for maintenance and that 3-monthly "1-week maintenance" might not be adequate to achieve an anticancer effect. This result, however, shows that it might be possible to decrease the number of maintenance instillations and further investigation is needed.

The completion rate of maintenance therapy in the SWOG trial was 16%,[6] whereas another study reported that only one of 111 patients completed seven cycles of maintenance therapy.[12] In contrast, a high completion rate of 75% was achieved in the present study. This is not only attributable to the small number of instillations for maintenance therapy, but also to a lower frequency and severity of ADR occurring during maintenance. In the EORTC prospective study, the majority of discontinuations as a result of adverse events were seen during induction therapy and the first 6 months of maintenance therapy, suggesting that BCG maintenance does not necessarily increase the occurrence of adverse events.[13] Patients who show good compliance to BCG treatment during the induction therapy can receive maintenance therapy safely.

Concerning cancer treatment, it is essential to evaluate treatment from the patient's point of view, besides the clinical outcome, and therefore QOL assessment was incorporated in the present study. Induction therapy did not deteriorate QOL and the emotional functioning scale improved significantly. It seems that patients' good acceptance of their disease state and treatment using BCG improved their own QOL. Furthermore, BCG maintenance did not deteriorate the QOL, which might be the reason for the good completion rate of maintenance treatment.

In conclusion, the present study, despite having a small scale, is a meaningful prospective investigation suggesting both the usefulness of BCG maintenance in patients with high-risk NMIBC and the probability that a schedule with fewer maintenance instillations would still be effective. The maintenance therapy prevented recurrence with fewer ADR and no deteriorations in QOL. Our future task is to continue exploring both the benefit and the optimal treatment schedule of maintenance therapy.

Acknowledgments

This study was supported by a grant from Japan BCG Laboratory Co. Ltd.

References

1 Lamm DL, Blumenstein BA, Crawford ED *et al.* A randomized trial of intravesical doxorubicin and immunotherapy with Bacillus Calmette-Guérin for transitional-cell carcinoma of the bladder. *N. Engl. J. Med.* 1991; **325**: 1205–9.
2 van der Meijden AP, Brausi M, Zambon V *et al.* Intravesical instillation of epirubicin, bacillus Calmette-Guérin and bacillus Calmette-Guérin plus isoniazid for intermediate and high risk Ta T1 papillary carcinoma of the bladder: a European Organization for Research and Treatment of Cancer Genito-Urinary Group randomized phase III trial. *J. Urol.* 2001; **166**: 476–81.
3 Sylvester RJ, van der Meijden APM, Lamm DL. Intravesical bacillus Calmette-Guérin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. *J. Urol.* 2002; **168**: 1964–70.
4 Babjuk M, Oosterlinck W, Sylvester R *et al.* EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder. *Eur. Urol.* 2008; **54**: 303–14.
5 Hall MC, Chang SS, Dalbagni G *et al.* Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update. *J. Urol.* 2007; **178**: 2314–30.
6 Lamm DL, Blumenstein BA, Crissman JD *et al.* Maintenance bacillus Calmette-Guerin immunotherapy for recurrent TA, T1 and carcinoma in situ transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study. *J. Urol.* 2000; **163**: 1124–9.
7 Böhle A, Bock PR. Intravesical bacille Calmette-Guérin versus mitomycin C in superficial bladder cancer: formal meta-analysis of comparative studies on tumor progression. *Urology* 2004; **63**: 682–6.
8 Aaronson NK, Ahmedzai S, Bergman B *et al.* The European Organization for Research and Treatment of Cancer QLQ-C30: a quality-of-life instrument for use in international clinical trials in oncology. *J. Natl. Cancer Inst.* 1993; **85**: 365–76.
9 Japanese Urological Association and the Japanese Society of Pathology. *General Rule for Clinical and Pathological Studies on Bladder Cancer*, 3rd edn. Kanehara Shuppan, Tokyo, 2001.
10 Akaza H, Hinotsu S, Aso Y *et al.* Bacillus Calmette-Guérin treatment of existing papillary bladder cancer and carcinoma in situ of the bladder. Four-year results. *Cancer* 1995; **75**: 552–9.
11 Koga H, Kuroda M, Kudo S *et al.* Adverse drug reactions of intravesical bacillus Calmette-Guerin instillation and risk factors of the development of adverse drug reactions in superficial cancer and carcinoma in situ of the bladder. *Int. J. Urol.* 2005; **12**: 145–51.

12 Decobert M, LaRue H, Harel F *et al.* Maintenance Bacillus Calmette-Guérin in high-risk nonmuscle-invasive bladder cancer. How much is enough? *Cancer* 2008; **113**: 710–16.
13 van der Meijden APM, Sylvester RJ, Oosterlinck W *et al.* Maintenance Bacillus Calmette-Guerin for Ta T1 bladder tumors is not associated with increased toxicity: results from European Organisation for Research and Treatment of Cancer Genito-Urinary Group Phase III Trial. *Eur. Urol.* 2003; **44**: 429–34.

Appendix

**BCG Tokyo Strain Study Group*: The institutions and their representative investigators of BCG Tokyo Strain Study Group are as follows: T Tsukamoto (Sapporo Medical University), T Suzuki (Hirosaki University), Y Arai (Tohoku University), Y Kitamura (Niigata Cancer Center Hospital), N Miyanaga (Tsukuba University), T Tobe (Chiba University), I Fukui (Cancer Institute Hospital of Japanese Foundation for Cancer Research), K Tomita (Tokyo University), Y Horiguchi (Keio University), K Asano (Jikei University), S Baba (Kitasato University), H Furuse (Hamamatsu University), H Sakagami (Anjo Kosei Hospital), Y Ono (Nagoya University), K Tozawa (Nagoya City University), K Fujimoto (Nara Medical University), H Nishiyama (Kyoto University), T Nomoto (Kyoto Prefectural University), M Usami (Osaka Medical Center for Cancer and Cardiovascular Diseases), T Shinka (Wakayama Medical University), S Kamidono (Kobe University), T Tsushima (Okayama University), A Terai (Kurashiki Central Hospital), Y Sumiyoshi (Shikoku Cancer Center), K Naito (Yamaguchi University), S Naito (Kyushu University), A Yamaguchi (Harasanshin Hospital), K Nomata (Nagasaki University).

The members of the Efficacy and Safety Evaluation Committee are as follows: K Koiso (Senpo Tokyo Takanawa Hospital), Y Matsumura (Hyogo Health Service Association), S Hinotsu (Tsukuba University).

Editorial Comment

Editorial Comment to Maintenance intravesical bacillus Calmette-Guérin instillation for Ta, T1 cancer and carcinoma *in situ* of the bladder: Randomized controlled trial by the BCG Tokyo Strain Study Group

In this paper, Koga *et al.* investigated the additional effect of a 1-year maintenance bacillus Calmette-Guérin (BCG) regime using four instillations of BCG Tokyo strain 80 mg at 3, 6, 9 and 12 months. The authors found a higher recurrence-free survival at 2 years in the maintenance group, although this did not reach statistical significance. Of the patients randomised to receive maintenance treatment, 75% completed the regime.[1]

The benefits of BCG maintenance in patients with high risk, non-muscle invasive, bladder cancer (NMIBC) have been confirmed by meta-analysis,[2] but it is well known that many patients never complete their full maintenance regime due to side-effects.[3]

We do not know the optimal schedule, length and indeed strain of maintenance BCG that produces the greatest reduction in recurrence rate with the fewest side-effects, but there is increasing evidence that a 1-year maintenance schedule might be the answer. Although the higher recurrence-free survival for the maintenance group did not reach statistical significance at 2 years, the Kaplan–Meier curves suggest that the effect might be more pronounced with a longer follow up. In addition, the majority of patients in this study had primary carcinoma *in situ*, which inherently carries a higher progression and recurrence risk than papillary high risk NMIBC, and this might also have reduced the size of any effect.

In conclusion, the authors are to be congratulated on demonstrating the feasibility of a reduced intensity maintenance schedule of four additional instillations over a 1-year period that appears to be well tolerated. Further work is necessary to answer the question: "How little BCG is enough to be effective?".

Hugh Mostafid FRCS(UROL) FEBU
Department of Urology,
North Hampshire Hospital,
Basingstoke, Hampshire, UK
mostafid@globalnet.co.uk

References

1 Koga H, Ozono S, Tsushima T *et al.* Maintenance intravesical basillus Calmette-Guérin instillation for Ta, T1 cancer carcinoma *in situ* of the bladder: Randomized controlled trial by the BCG Tokyo Strain Study Group. *Int. J. Urol.* 2010; **17**: 759–66.

Cancer Science
The official journal of the Japanese Cancer Association

Fibroblast growth factor receptor 3 mutation in voided urine is a useful diagnostic marker and significant indicator of tumor recurrence in non-muscle invasive bladder cancer

Makito Miyake,[1,8] Kokichi Sugano,[1,9] Hitomi Sugino,[1] Kazuho Imai,[1] Eri Matsumoto,[1] Koshi Maeda,[2] Shinich Fukuzono,[2] Hiroki Ichikawa,[3] Kiyotaka Kawashima,[3] Kaoru Hirabayashi,[4] Tetsuro Kodama,[5] Hiroyuki Fujimoto,[6] Tadao Kakizoe,[6] Yae Kanai,[7] Kiyohide Fujimoto[8] and Yoshihiko Hirao[8]

[1]Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, 4-9-13 Yonan, Utsunomiya; [2]Hitachi High-Technologies Corporation, Hitachinaka, Ibaraki; [3]Department of Urology, [4]Department of Clinical Laboratory, [5]Director, Tochigi Cancer Center Hospital, Utsunomiya, Tochigi; [6]Department of Urology, National Cancer Center Hospital, [7]Pathology Division, National Cancer Center Research Institute, Tokyo; [8]Department of Urology, Nara Medical University, Kashihara, Nara, Japan

(Received May 06, 2009/Revised August 17, 2009/Accepted August 23, 2009/Online publication October 14, 2009)

The fibroblast growth factor receptor (FGFR)-3 gene encodes a receptor tyrosine kinase that is frequently mutated in non-muscle invasive bladder cancer (NMIBC). A sensitive and quantitative assay using peptide nucleic acid-mediated real-time PCR was developed for detecting *FGFR3* mutations in the urine samples and evaluated as a molecular marker for detecting intravesical recurrence of NMIBC in patients undergoing transurethral resection of bladder tumor. *FGFR3* mutation was examined in tumor tissues and serially taken pre- and postoperative urine sediments in 45 NMIBC patients with a median follow up of 32 months. *FGFR3* mutations were detected in 53.3% (24/45) of primary tumor tissues, among which intravesical recurrence developed in 37.5% (9/24) of cases. *FGFR3* mutation in the primary tumor was not a significant prognostic indicator for recurrence, while the proportion of *FGFR3* mutation (i.e. tumor cellularity was ≥11%) in the preoperative urine sediments was a significant indicator for recurrence in patients with *FGFR3* mutations in the primary tumors. *FGFR3* mutations were detected in 78% (7/9) of postoperative urine samples from recurrent cases with *FGFR3* mutations in the tumor, while no mutations were detected in the urine of 15 non-recurrent cases. Urine cytology was negative in all cases with *FGFR3* mutations in the primary tumors, while the sensitivity of cytological examination was as high as 56% (5/9) in cases showing wild-type *FGFR3* in the primary tumors. Urine *FGFR3* mutation assay and cytological examination may be available in the future as complementary diagnostic modalities in postoperative management of NMIBC. (*Cancer Sci* 2010; 101: 250–258)

Urothelial carcinoma (UC) is a histological subtype accounting for more than 90% of all bladder cancers, and there are 357 000 new cases every year worldwide.[1] Bladder UCs are generally divided into two groups for clinical management, depending on the pathological stage. Most of the newly diagnosed UCs are non-muscle invasive bladder cancer (NMIBC; i.e., pTa or pT1), and the initial treatment is transurethral resection of bladder tumor (TURBT). After the initial TURBT, the patients undergo intensive surveillance by cystoscopic examination at regular intervals; usually every 3 months, because up to 70% of these patients will experience intravesical recurrence, and 10–30% of the lesions will progress to life-threatening muscle-invasive disease (≥pT2).[2] Cystoscopy is an inconvenient, invasive, and expensive diagnostic modality, but currently it is the gold standard for detecting intravesical recurrence in the postoperative follow up. Although urine bound diagnostic tests including urinary cytology, nuclear matrix protein (NMP)22, and bladder tumour antigen (BTA) tests are used in the management after TURBT or bladder cancer screening, their usefullness is limited due to their poor sensitivity or specificity.[3] In previous reports, various molecular markers detectable in urine have been considered as a useful and non-invasive clinical assay improving the sensitivity of conventional tests.[4–10] In urine-based detection assays, contamination with normal urothelium or leucocytes can mask the signals of targeted somatic mutations.[11]

Fibroblast growth factor receptor (FGFR)-3 belongs to a family of structurally related tyrosine kinase receptors (FGFR1–4), and plays important roles in many biological processes including embryogenesis, proliferation, differentiation, and angiogenesis.[12] Recent reports have demonstrated that constitutively activated *FGFR3* mutations exist in more than 50% of primary bladder UC.[13] *FGFR3* mutations are especially prevalent in the low-grade papillary tumors (pTa/G1), but they are infrequent in high-grade or high-stage UC.[13,14] *FGFR3* mutation in urine sediments may be a suitable biomarker for detection of low-grade and low-stage UC. Previous studies revealed that mutation of *FGFR3* in the voided urine can be detected at high sensitivity in patients with *FGFR3*-mutated bladder UC.[15–17] However, there is no report validating the feasibility and usefulness of detecting *FGFR3* mutation in the voided urine samples by serial determinations during follow up after TURBT. Recently, we have reported an assay protocol for detecting *FGFR3* mutations in bladder tumor tissues and urine sediments by peptide nucleic acid (PNA)-mediated real-time PCR clamping assay.[17] In PNA-mediated PCR clamping, PNA is designed to anneal to a wild-type DNA sequence and inhibits the annealing of PCR primer to the wild-type alleles, resulting in preferential amplification of the mutated alleles. With 50 ng of genomic DNA as a template, this method allows sensitive and quantitative detection of the *FGFR3* mutations in mutational hotspots in exons 7, 10, and 15 in bladder cancer. In the present study, we modified the protocol of the PNA-mediated PCR clamping assay to achieve quantitative detection of the *FGFR3* mutations present in the urine samples at a concentration of 1% in only 1 ng of genomic DNA available as a template for PCR. With this the revised protocol, we assessed the usefulness of *FGFR3* mutations as a

[9]To whom correspondence should be addressed.
E-mail: ksugano@tcc.pref.tochigi.lg.jp

doi: 10.1111/j.1349-7006.2009.01334.x

diagnostic modality in the voided urine samples for the postoperative management of NMIBC. This is considered the first report addressing the significance of *FGFR3* mutations in preoperative urine sediments as a novel indicator predicting the risk of intravesical recurrence of NMIBC.

Materials and Methods

Subjects and collection of the tumor tissues and voided urine samples after the initial TURBT. The patients undergoing TURBT from April 2002 through March 2005 at the Departments of Urology at Tochigi Cancer Center Hospital and Nara Medical University Hospital were enrolled in this study. All participants had received study information and signed a written informed consent form. The voided urine samples before the initial TURBT were taken from the patients. The resected tumors were examined histologically and staged and graded according to the 2002 TNM classification and the 1973 World Health Organization (WHO) classification systems, respectively.[18,19] A total of 45 subjects with NMIBC were eligible for the study and were followed up until the histological diagnosis of tumor recurrence or up to 3 years postoperatively by routine cystoscopy and urine cytological examination. The median follow-up period was 32 months (range 4–36 months). The patients were monitored by routine cystoscopy and urine cytology at 1, 3, 6, 9, 12, 15, 18, 21, 24, 30, and 36 months after the initial TURBT. Intravesical recurrence was confirmed by histological diagnosis of tumor tissues obtained during TURBT for recurrence. The voided urine samples were divided and subjected to urine cytology and DNA extraction for gene testing. The urine samples were stored at −20°C until DNA extraction.

DNA extraction and measurement of DNA concentration. DNA extraction from the tumor tissues and peripheral blood lymphocytes (PBL) was carried out as described previously.[6] DNA extraction from the urine samples was carried out with the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, the urine sample in a 50-mL tube was centrifuged at 180*g* for 5 min. The cell pellet was digested by Qiagen protease and subjected to DNA extraction by column centrifugation. In the final step, DNA was eluted from the column in 150 μL of the elution buffer. The genomic DNA concentration was determined by ultraviolet measurement using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For analysis of samples with DNA concentrations less than 50 ng/μL, DNA concentration was quantified by real-time PCR using LightCycler (Roche Diagnostics, Mannheim, Germany). Quantification was carried out with the same primer pairs used for PNA-mediated real-time PCR clamping for amplification of *FGFR3* exon 7.[17] Serially diluted assay standards were prepared by adjusting the genomic DNA concentrations to 100, 10, 1, and 0.1 ng/μL. DNA samples and assay standards were subjected to real-time PCR in a 20-μL reaction volume containing genomic DNA, 10 picomole of each primer, and 10 μL of QuantiTect PCR master mix (Qiagen) containing SYBR Green I dye. The conditions of real-time PCR are described in Table 1. DNA concentration was calculated from the crossing points (CP) of the assay standards and samples according to the fit points method on LightCycler Data Analysis software version 3.5 (Roche Diagnostics corporation).

PNA-mediated pre-main amplifier method for the low-copy number DNA template. Previously, we reported a PNA-mediated real-time PCR clamping assay for detection of *FGFR3* mutations.[17] This method enabled sensitive and reproducible detection of *FGFR3* mutations in cases where 50 ng of genomic DNA were available as the template for PCR. In the PNA-mediated PCR-clamping, the chance of nucleotide misincorporation to the PNA binding sequence increases in reverse correlation with the amount of template DNA. When the amount of template DNA was 1 ng in genomic DNA (equivalent to 300 copies), mutations were hardly distinguishable from those caused by misincorporation of dNTPs. To overcome this pitfall, we modified the assay protocol to detect *FGFR3* mutations at a concentration of 1% (three copies) in 1 ng (300 copies) of the template genomic DNA. We called the newly established method as PNA-mediated pre-main amplifier (PPA), which consisted of two steps of amplification (Fig. 1). Low-copy number DNA template was amplified by the pre-amplifier step and then set on the main amplifier to perform the PNA-mediated real-time PCR clamping. Pre-amplification was carried out in a PCR tube using DNA Engine Dyad Thermal Cycler (MJ Research, Watertown, MA, USA) in 20-μL aliquots consisting of 1 ng of genomic DNA, 10 μL of QuantiTect PCR master mix, and 10 picomole of each primer. The sequences of primer pairs were as reported previously.[17] Conditions of the thermal cycling in the pre-amplifier step were as follows: denaturing at 95°C for 15 min, amplification of seven cycles consisting of heat denaturation at 94°C for 15 s, annealing at 64°C (exon 7), 58°C (exon 10), and 60°C (exon 15) for 20 s, and extension at 72°C for 20 s. After final cooling to 4°C, 5 μL of the solution containing 2.5 μL of QuantiTect PCR master mix and 2.5 μL of PNA solution were added and mixed by gentle pipetting. The sequences of PNA and the final concentrations are listed in Table 1. Of 25 μL of the mixed solution, 20 μL was transferred to a capillary tube for the LightCycler and set on the main amplifier performing the real-time PCR (Table 1). CP of PPA were determined by the fit points method.

Detection of *FGFR3* mutations in the tumor tissues and urine samples. The assay standards for mutation analysis of each exon were prepared as described previously.[17] In the clinical

Table 1. Sequences of PCR primers and peptide nucleic acid (PNA), and PCR conditions

Real-time PCR	Sequence of primers and PNA	PNA concentration (μM)	Cycle no.	PNA binding step (°C)	Annealing step (°C)
DNA quantification	5′-TGA GCG TCA TCT GCC CCC ACA GAG-3′ (sense) 5′-GGG CCC ACC TTG CTG CCA TTC A-3′ (antisense)	–	45	–	64
Main amplifier for exon 7	5′-TGA GCG TCA TCT GCC CCC ACA GAG-3′ (sense) 5′-GGG CCC ACC TTG CTG CCA TTC A-3′ (antisense) H2N-AGC GCT CCC CGC ACC-N2H (PNA)	0.4	45	72	64
Main amplifier for exon 10	5′-CCA GGC CTC AAC GCC CAT GTC TTT-3′ (sense) 5′-ACC CCG TAG CTG AGG ATG CCT GCA-3′ (antisense) H2N-CAT ACA CAC TGC CCG C-N2H	1	45	67	58
Main amplifier for exon 15	5′-GCA ATG TGC TGG TGA CCG AG-3′ (sense) 5′-CGG GCT CAC GTT GGT CGT CT-3′ (antisense) H2N-GGT CGT CTT CTT GTA GT-N2H	2	45	70	60

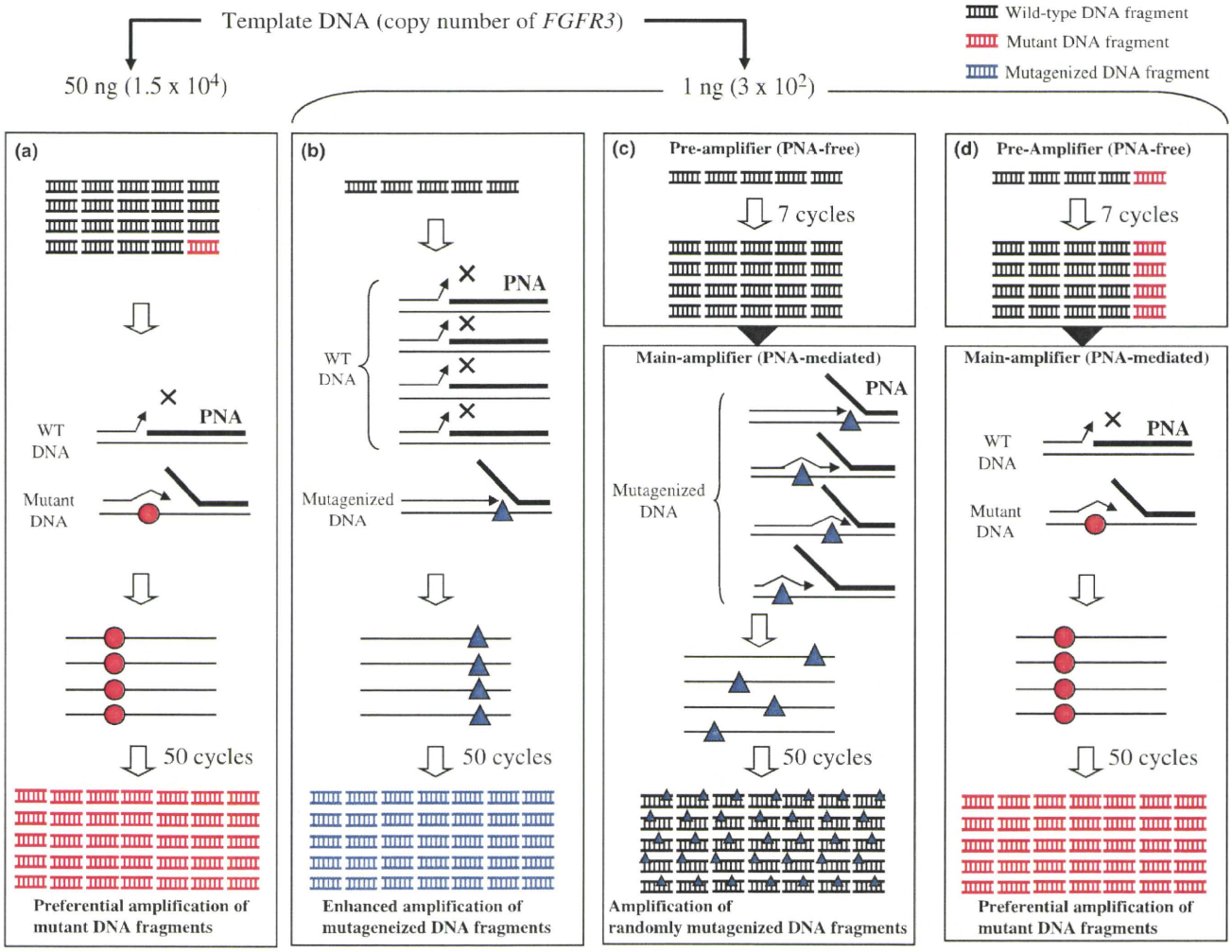

Fig. 1. Schematic diagram of the one-step and two-step peptide nucleic acid (PNA)-mediated PCR clamping. The feasibility of PNA-mediated PCR clamping was highly influenced by the amount of the template DNA. (a) Fifty nanograms of genomic DNA containing a low proportion of mutated DNA was used as a template. PNA-mediated PCR enabled preferential amplification of the mutated DNA, leading to enrichment of the mutated DNA fragments. The red circle indicates a mutated DNA sequence. (b) One nanogram of genomic DNA containing only wild-type DNA was used as the template. Misincorporation of dNTPs occurred in the sequences of the PNA-binding site, due to a failure in DNA synthesis brought about by DNA polymerase. When the nucleotide misincorporation (blue triangle) occurred in the early cycles of PNA-mediated PCR, a mutagenized sequence was subjected to subsequent amplification. (c) One nanogram of the template DNA containing only wild-type DNA was used for the PNA-free pre-amplifier step prior to the PNA-mediated main-amplifier. Seven PCR cycles of the pre-amplifier step generated sufficient copies of fibroblast growth factor receptor (*FGFR*)-3 DNA fragments, which were used as the template for the main-amplifier. The PNA-mediated reaction produced a randomly mutagenized DNA sequence that could slip from PNA clamping. However, all of these mutagenized fragments resulted in dispersion of mutagenesis signals and were scarcely detectable in the direct sequencing analysis. (d) One nanogram of genomic DNA with a low proportion of mutant DNA was used as the template. The PNA-free pre-amplifier increased the copy numbers of the *FGFR3* molecules as a whole, leading to a successful preferential amplification of the mutated DNA fragments in the main-amplifier. The black, red, and blue fragments indicate the wild-type, mutated, and mutagenized DNA fragments, respectively.

samples with DNA concentrations of ≥50 ng/μL, mutation analyses were carried out according to the one-step assay using 50 ng of genomic DNA as the template.[17] In the samples with DNA concentrations ranging from 0.125 to 50 ng/μL, a modified protocol was adapted using 1 ng of genomic DNA as the template. In each run, we defined CP of the assay standard corresponding to 1% tumor cellularity as the minimal detectable dose for *FGFR3* mutations. Accordingly, a sample showing CP less than that of the 1% assay standard was considered mutation positive and subjected to direct sequencing to identify the mutational types.[17] The tumor cellularities of the mutation-positive samples were determined by a regression analysis using a standard curve obtained from 100, 10, and 1% assay standards. The samples with DNA concentrations less than 0.125 ng/μL were regarded as unavailable samples unless they could be enriched in DNA concentration.

Statistical analysis. Statistical analyses and drawing figures were done using PRISM software version 4.00 (GraphPad Software, Inc., San Diego, CA, USA). Student's *t*-test, Chi-square test, and Fisher's exact test were used to analyze the correlations between the clinicopathological variables and *FGFR3* mutational status in the primary tumors. Recurrence-free survival curves were plotted according to the Kaplan–Meier method, and the log-rank test was applied for statistical significance. A receiver operating characteristic (ROC) curve was used to define the optimal cut-off value of tumor cellularity in the urine sediments. The non-parametric variables were analyzed by the Mann–Whitney *U*-test. A *P*-value of <0.05 was considered significant.

doi: 10.1111/j.1349-7006.2009.01334.x

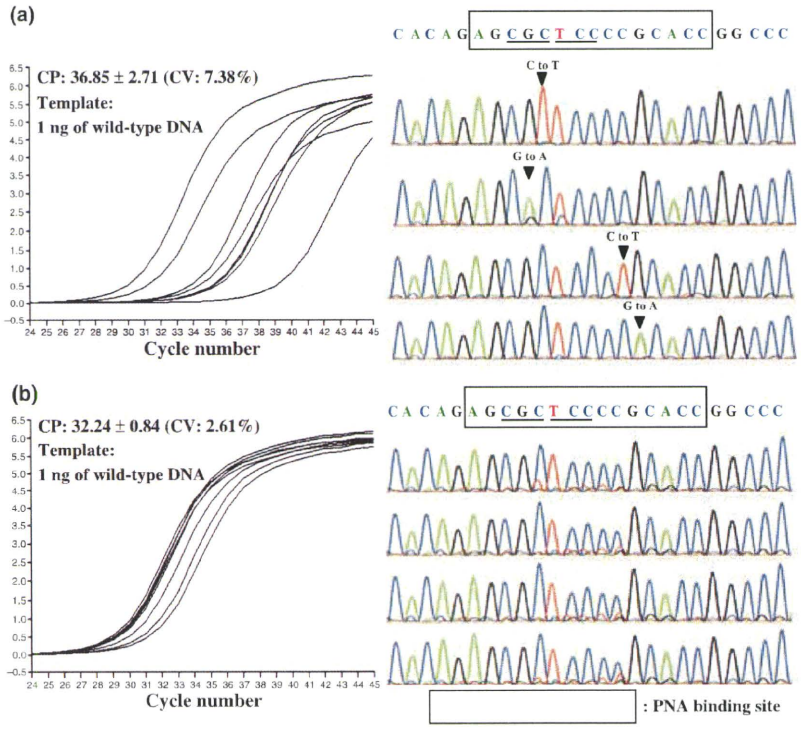

Fig. 2. Two-step peptide nucleic acid (PNA)-mediated real-time PCR clamping decreased the variance of crossing points (CP) and avoided the enhanced misincorporation of dNTPs. In the experiment for fibroblast growth factor receptor (*FGFR*)-3 exon 7, 1 ng of genomic DNA containing only wild-type *FGFR3* was amplified in octaplicate (n = 8) by the (a) one-step and (b) two-step PNA-mediated real-time PCR. The PNA-mediated pre-main amplifier method consisted of seven cycles of pre-amplification followed by 45 cycles of the main amplification step. The amplification curves and representative results of direct sequencing analysis of the PCR products are shown in the left and right panels, respectively. The mean CP ± SD and coefficient of variance (CV) are shown above the amplification curves. (a) Arrowheads indicate disincorporated nucleotides caused by PNA clamping. The uppermost sequence indicates the wild-type *FGFR3* in exon 7. The PNA binding site is surrounded by a rectangle. Codons 248 and 249 are underlined.

Results

Optimization of PPA for detection of *FGFR3* mutations in low-copy number DNA template. The number of PCR cycles in the pre-amplifier step was critical for sensitive detection of *FGFR3* mutations in the low-copy number DNA template. In a preliminary experiment to optimize the number of PCR cycles in the pre-amplifier step, the concentration of *FGFR3* mutation in the sample was adjusted to either 1 or 0% and the difference in CP was maximal when seven cycles of PCR were used in the pre-amplification step (P = 0.001). In this condition, we compared the coefficients of variation (CV) between the one-step and two-step assays using 1 ng of wild-type genomic DNA as the template (Fig. 2a,b left). The assay CV of CP in the PPA method was much smaller than that of the one-step assay (2.61 vs 7.38%, respectively). The sequencing analysis of the amplified DNA fragments in the one-step assay revealed point mutations caused by nucleotide misincorporation virtually in all samples (Fig. 2a right), whereas those amplified by the PPA assay showed no recognizable mutations except for a slight increase in the background signals. These results indicated that the PPA method circumvented the chance of a nucleotide misincorporation and minimized the CV of the CP for wild-type DNA or 0% standard (Fig. 2b right). In this condition, the assay standards with 100, 10, and 1% mutations in exon 7 of *FGFR3* and 0% (wild type) were amplified by the PPA method using 1 ng of DNA template, and the results were compared with those of the one-step assays. The CPs of the assay standards were statistically significant between each other (Fig. 3a) and direct sequencing analysis of the 1% standard revealed that all of eight samples showed S249C mutation (TCC → TGC) in exon 7. These results demonstrated that the mutations were reliably detected in the samples containing ≥1% mutated DNA using only 1 ng of DNA template, and that the PPA method overcame the limitation of our prior study.

In analysis of exons 10 and 15, seven amplification cycles in the pre-amplifier step were used to detect mutations in the samples containing ≥1% mutated DNA using 1 ng of genomic DNA as the template (Fig. 3b,c).

Correlation of *FGFR3* mutations with the clinicopathological characteristics in NMIBC. In analysis of *FGFR3* mutations in 45 NMIBC samples, 24 (53.3%) tumors harbored activating mutations of *FGFR3*, and their correlations with the clinicopathological variables are summarized in Table 2. No variables showed significant correlation with *FGFR3* mutations. Mutations were detected in six different codons. Mutations affecting the extracellular domain (exon 7) or transmembrane domain (exon 10) accounted for 95.8% (23/24) (Table 3). Intravesical recurrence was detected in 18 of 45 subjects (40%). The clinicopathological variables of the primary tumors, such as tumor stage, histological grade, tumor size, multiplicity, presence of carcinoma *in situ* lesion, and *FGFR3* mutational status, did not correlate with the intravesical recurrence (Table 4).

Clinical usefulness of detecting *FGFR3* mutation in the urine sediments. A total of 429 voiding urine samples were taken from 45 cases, among which 61 samples were preoperative urine samples consisting of 35 from recurrent and 26 from non-recurrent cases (Table 5). The remaining 368 urine samples were obtained serially during follow up, among which 93 samples were from recurrent cases and 275 samples were from non-recurrent cases. The concentrations of genomic DNA extracted from the urine samples were quantified in all samples prior to the assay. Of 429 urine samples, 114 (26.6%) were not available for the assay because their DNA concentrations were <0.125 ng/μL. A total of 315 samples (73.4%) were subjected to the *FGFR3* mutation detection assay. They were subjected to either the conventional PNA-mediated real-time PCR clamping assay or PPA method depending on their DNA concentrations.

Risk of intravesical recurrence in patients showing *FGFR3* mutation in tumor tissues and urine sediments. In 21 of 24 cases with *FGFR3* mutation in primary tumors, genomic DNA samples extracted from preoperative urine sediments before the initial TURBT were available for mutation detection assay. The sensitivity of *FGFR3* mutation in the urine samples was 62%

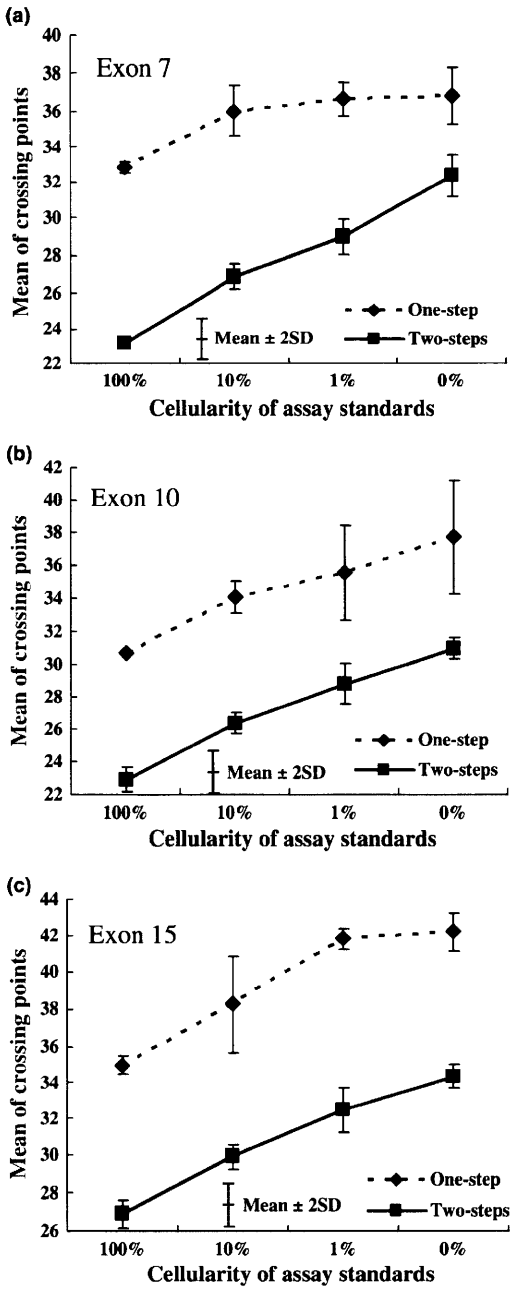

Fig. 3. Validation for sensitive detection of fibroblast growth factor receptor (*FGFR*)-3 mutation in trace amounts of the template DNA. (a) One nanogram of genomic DNA of four assay standards with 0, 1, 10, and 100% of tumor cellularity was amplified in octaplicate samples (n = 8) under the conditions of the two-step assays comprising seven amplification cycles of the peptide nucleic acid (PNA)-free pre-amplifier, followed by PNA-mediated real-time PCR clamping for exon 7 of the *FGFR3* gene (solid lines). The data from the one-step assay are shown for comparison (dashed lines). The mean crossing points (CP) are plotted and the error bars represent 2 SD. Similar experiments were carried out to validate the assay protocols for (b) exon 10 and (c) exon 15.

(13/21), and their mutational types coincided with those of the primary tumors in all cases. In 21 cases showing wild-type *FGFR3* in primary tumor tissues, the preoperative urine samples were available for the assay in 13 cases and no mutations were found in these samples, showing the specificity of 100% (0/13). Tumor cellularities in the preoperative urine sediments were significantly higher in the recurrent cases than in the non-recurrent cases (Fig. 4a; P = 0.008). An ROC curve analysis was performed to define the optimal cut-off value of tumor cellularity in the preoperative urine sediments. The area under the curve (AUC) was 0.847 (95% confidence interval, 0.669–1.026), and the cut-off value with optimal sensitivity and specificity was defined as 11% (Fig. 4b).

FGFR3 mutational status in the primary tumors was not a significant predictor of intravesical recurrence after TURBT

Table 2. Clinicopathological characteristics and fibroblast growth factor receptor (*FGFR*)-3 mutation status

Variables	Total	*FGFR3* status			*P*-value
		Wild-type	Mutation	% of mutation	
Age (years)					
Mean ± SD	63.0 ± 11.2	63.1 ± 12.7	62.8 ± 9.6	–	0.98
Range	36–80	36–80	45–78	–	
Sex					
Male	35	15	20	57	0.27
Female	10	6	4	40	
Smoking history					
Present	29	11	18	62	0.11
Absent	16	10	6	38	
Tumor size (diameter, cm)					
<1	10	4	6	60	0.73
1–3	25	13	12	48	
3<	10	4	6	60	
Multiplicity					
Solitary	27	12	15	56	0.71
2–3	12	5	7	58	
≥4	6	4	2	33	
Pathological stage					
pTa	19	7	12	63	0.26
pT1	26	14	12	46	
Tumor grade					
G1	6	3	3	50	0.97
G2	35	16	19	54	
G3	4	2	2	50	
Concomitant CIS					
Present	3	2	1	33	0.45
Absent	42	19	23	55	
BCG therapy					
No	40	19	21	53	0.87
Treated	5	2	3	60	
Total	45	21	24	53	

BCG, Bacille Calmette Guerin; CIS, carcinoma *in situ*.

Table 3. Fibroblast growth factor receptor (*FGFR*)-3 mutational types detected in this study

FGFR3	Mutational type		*n*	%
	Codon	Nucleotide		
Exon 7	R248C	CGC → TGC	4	16.7
	S249C	TCC → TGC	8	33.3
Exon 10	G372C	GGC → TGC	1	4.2
	S373C	AGC → TGC	1	4.2
	Y375C	TAT → TGT	9	37.5
Exon 15	K652E	AAG → GAG	1	4.2
Total			24	100

doi: 10.1111/j.1349-7006.2009.01334.x

Table 4. Correlation of the stage, histological grade, tumor size, multiplicity, concomitant CIS and fibroblast growth factor receptor (*FGFR*)-3 mutations in the tissue with intravesical tumor recurrence

Variables	Total	Recurrent	Non-recurrent	*P*-value*
No. subjects	45	18	27	
Stage				
pTa	19	8	11	1.00
pT1	26	10	16	
Grade				
G1	6	1	5	0.43
G2	35	15	20	
G3	4	2	2	
Tumor size (cm)				
<3	35	12	23	0.14
≥3	10	6	4	
Multiplicity				
Solitary	27	10	17	0.45
Multiple	18	8	10	
Concomitant CIS				
Absent	42	16	26	0.25
Present	3	2	1	
FGFR3 mutations in the tumor tissues				
Wild-type	21	9	12	0.89
Mutation	24	9	15	

CIS, carcinoma *in situ*; *logrank test.

(Table 4). In cases harboring *FGFR3* mutations in the primary tumor, the levels of *FGFR3* mutations in the preoperative urine sediments significantly correlated with the 3-year recurrence-free survival rates (83.3 vs 22.2%) (Fig. 4c), whereas the results of preoperative urine cytology did not correlate with the recurrence-free survival.

Serial determination of *FGFR3* mutations in the voided urine samples during the follow-up period after the initial TURBT. *FGFR3* mutations in serially obtained urine samples were assayed quantitatively in the postoperative follow-up period. Low-copy number DNA samples were amplified by the PPA method using 1 ng of genomic DNA as the template. In 21 cases harboring *FGFR3* mutations in primary tumors, assay results were plotted on a 3-D line chart (Fig. 5). The preoperative sensitivities of urine *FGFR3* mutations in the recurrent and non-recurrent cases were 88.9% (8/9) and 41.7% (5/12), respectively. *FGFR3* mutations were not detected 1 month after the initial TURBT in all cases. *FGFR3* mutations in the postoperative urine samples were detected in 7 of 9 (78%) recurrent cases. In two cases, mutations preceded cystoscopical detection of tumor relapse by 6 and 9 months, respectively. In analysis of the non-recurrent cases with *FGFR3* mutation in primary tumors, the pre- and postoperative urine samples were available for the assay in 12 cases and no cases were positive for *FGFR3* mutations except for five urine samples obtained before the initial TURBT. The frequencies of *FGFR3* mutations in the postoperative urine samples were compared with the results of cytological examinations between recurrent and non-recurrent cases (Table 6). The sensitivity and specificity of *FGFR3* mutations in the postoperative urine samples were defined as the positive or negative rates of *FGFR3* mutations in recurrent or non-recurrent cases, respectively. In cases with *FGFR3* mutation in the primary tumors, the sensitivity and specificity of urine *FGFR3* mutations in the recurrent and non-recurrent cases were 78% (7/9) and 100% (15/15), respectively. Cytological examination was negative in all cases with *FGFR3* mutations in the tumors. In analysis of cases with wild-type *FGFR3* in the primary tumors, *FGFR3* mutations were not detected in the postoperative urine samples in the recurrent cases (0/9) except for one non-recurrent case (1/12), indicating sensitivity of 0% and specificity of 92% (11/12). In analysis of non-recurrent cases showing wild-type *FGFR3* in the tumor, S249C mutation was detected in one case at the 36th postoperative month with tumor cellularity of 2.1% (data not shown). This was the last session in postoperative follow up and no further information was available as to the clinical outcome in this case, although no abnormality was detected by cystoscopical and cytological examinations carried out simultaneously. The sensitivity and specificity of the cytological examination were 56% (5/9) and 100% (12/12) in those with wild-type *FGFR3* in their primary tumors. When they were used in combination, the assay sensitivity, specificity, and diagnostic accuracy were 67% (12/18), 96% (26/27), and 84% (38/45) (Table 6). It was elucidated that these two diagnostic modalities may be used as a complementary approach to the postoperative management of NMIBC.

Discussion

In the PNA-mediated real-time PCR clamping, low yields of DNA from the urine samples seem to be a major limiting factor to define the assay sensitivity.[17] To increase the copy numbers of the targeted gene in the template DNA, we added a pre-amplification step comprising seven cycles of PCR prior to the

Table 5. Correlation of DNA concentration and fibroblast growth factor receptor (*FGFR*)-3 mutations in the urine samples with tumor recurrence

Variables	Total	Recurrent	Non-recurrent	*P*-value
Collected urine samples	429	128	301	
Preoperative	61	35†	26	
Follow-up	368	93	275	
DNA concentration (ng/μL)				
Total				
50≦	83 (19.3%)	23 (18.0%)	60 (19.9%)	0.885
0.125–50	232 (54.1%)	71 (55.5%)	161 (53.5%)	
0.125<	114 (26.6%)	34 (26.6%)	80 (26.6%)	
Mean ± SD (ng/μL)	53.8 ± 187.6	36.2 ± 98.1	61.6 ± 214.3	
Detection of *FGFR3* mutation in urine sediments				
No. samples available for the assay	315	94	221	
Mutated	26 (8.3%)	20 (21.3%)	6 (2.7%)	<0.0001
WT	289 (91.7%)	74 (78.7%)	215 (97.3%)	

†Preoperative urine samples consisted of samples obtained in the initial transurethral resection of bladder tumor (n = 18) and transurethral resection of bladder tumor for recurrence (n = 17).

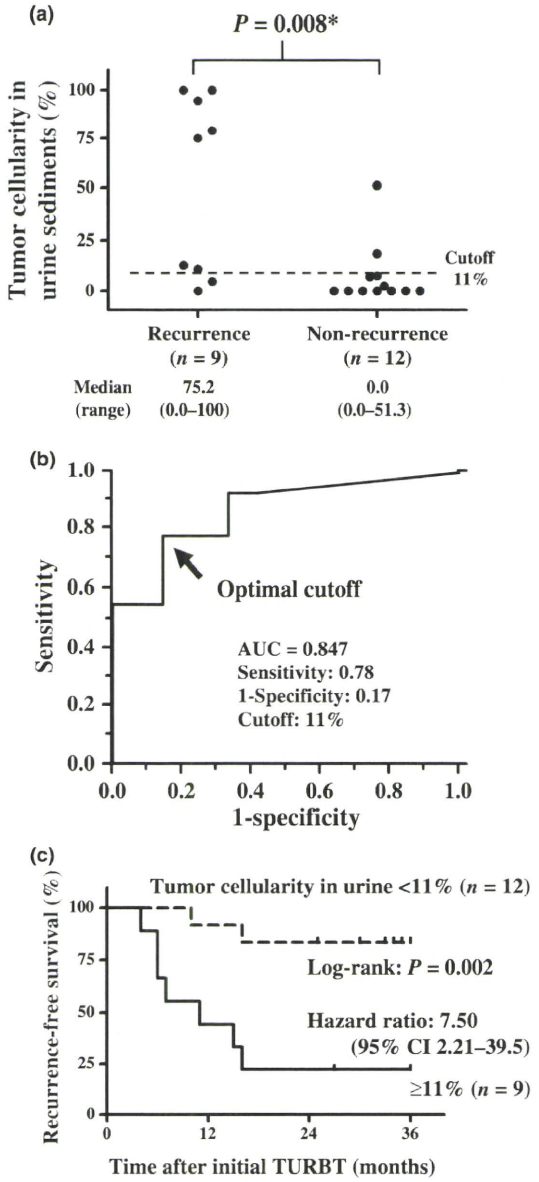

Fig. 4. The tumor cellularity in the preoperative urine predicted the risk of intravesical recurrence in patients with fibroblast growth factor receptor (*FGFR*)-3-mutated bladder urothelial carcinoma (UC). (a) The tumor cellularities in the preoperative urine sediments were plotted in the recurrent (n = 9) and non-recurrent (n = 12) groups of 21 cases with *FGFR3*-mutated bladder UC. The dashed line indicates the optimal cut-off point (≥11% and <11%) as determined with receiver operating characteristic (ROC) curve analysis shown in (b); *Mann–Whitney *U*-test. (b) A receiver operating characteristic curve was generated to define the optimal cut-off value of tumor cellularity in the preoperative urine sediments. The optimal cut-off value for predicting the intravesical recurrence was defined as the point closest to the upper-left corner of the graph (black arrow). (c) Recurrence-free survival curves according to the cut-off value (≥11% and <11%) of tumor cellularity in the preoperative urine sediments are shown. AUC, area under the curve; CI, confidence interval.

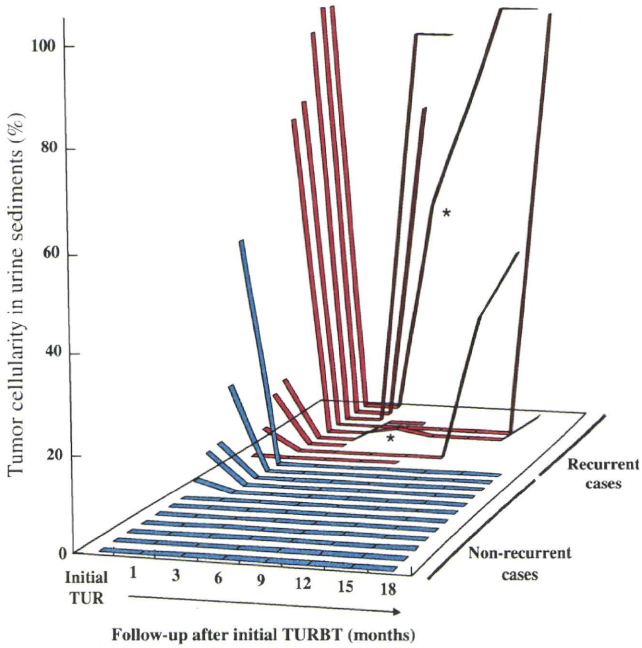

Fig. 5. The time-course analysis of tumor cellularity in the voided urine sediments in cases with mutated-fibroblast growth factor receptor (*FGFR*)-3. Tumor cellularities serially determined after initial transurethral resection of bladder tumor (TURBT) are plotted on 3-D line chart. All cases had *FGFR*3 mutations in their tumor tissues. Red and blue lines indicate the data of recurrent (n = 9) and non-recurrent (n = 12) cases, respectively. Two asterisks indicate the events of positive results prior to the cystoscopical detection of recurrent tumors. In recurrent cases, the last point of serial determination indicates tumor cellularity in the preoperative urine sediments obtained before second TURBT.

PNA-mediated real-time PCR clamping. This modified assay protocol achieved a sensitive method detecting *FGFR3* mutations at concentrations of ≥1% in 1 ng of the template DNA. One nanogram of genomic DNA with 1% of *FGFR3* mutations corresponds to as few as three copies of mutated *FGFR3*. Estimated from Poisson distribution, the probability of missing mutated FGFR3 molecules was 4.98% on sampling the aliquot containing *FGFR3* mutations at the concentration of 1% in 1 ng of the genomic DNA. The probabilities of missing mutated DNA molecules in the template increased up to 13.5% for two copies and 35.8% for a single copy, resulting in frequent false negatives. These assumptions suggested that 1% of tumor cellularity in 1 ng of the template DNA is actually the minimal detectable dose in the assay.

We developed a quantitative method to determine the proportion of *FGFR3* mutations in the sample as tumor cellularity and this approach disclosed the correlation between tumor cellularity and intravesical recurrence of NMIBC.

Assessing the risk for intravesical recurrence and progression after TURBT is another major concern in the clinical management of NMIBC. Many efforts have been reported to establish any molecular alterations in tumor tissues as prognostic markers.[2,20] Although some earlier studies have analyzed *FGFR3* mutation as a potential prognostic marker, the true prognostic value of *FGFR3* mutation is still controversial.[21–23] Quantitative analysis revealed that the tumor cellularity in the preoperative urine sediments strongly correlates with the tumor recurrence (P = 0.008). Moreover, the ROC analysis of the preoperative urine sediments determined the optimal cut-off value of *FGFR*3 mutations (11%) in the urine sediments that differentiates between the recurrent and non-recurrent tumors (Fig. 4). Using this cut-off value, the sensitivity, specificity, and diagnostic accuracy for detecting tumor recurrence were 77.8, 83.3, and 80.9%, respectively. This is the first report elucidating that the *FGFR3* mutational status in the urine sediments may serve as a

doi: 10.1111/j.1349-7006.2009.01334.x

Table 6. Association of fibroblast growth factor receptor (*FGFR*)-3 mutational status in primary tumors with cytological examination and/or *FGFR3* mutational status in voided urine samples

Assay	*FGFR3* status in primary tumor	Recurrent cases		Non-recurrent cases		Assay performance			
		Positive	Negative	Positive	Negative	Sensitivity (%)	Specificity (%)	Accuracy (%)	*P*-value
FGFR3 assay	Mutated	7	2	0	15	78	100	92	0.0001
	Wild-type	0	9	1	11	0	92	52	1.0000
	Total	7	11	1	26	39	96	73	0.0042
Urine cytology	Mutated	0	9	0	15	0	100	63	NA
	Wild-type	5	4	0	12	56	100	81	0.0062
	Total	5	13	0	27	28	100	71	0.007
Combination†	Mutated	7	2	0	15	78	100	92	0.0001
	Wild-type	5	4	1	11	56	92	76	0.0464
	Total	12	6	1	26	67	96	84	<0.0001

†Positivity is defined by the positive result in either urine cytology or FGFR3 mutation detection assay. NA, not analyzed.

prognostic indicator for tumor recurrence in NMIBC (Fig. 4c), indicating that cell detachment or exfoliation into the urine strongly correlates with the tumor recurrence.

Sequential analyses of the urine samples taken after the initial TURBT revealed intravesical recurrence in almost 78% (7/9) of the recurrent cases showing *FGFR3* mutations in the primary tumors, while the cytological examination of the same sample showed no positive results (Table 6). In two cases, *FGFR3* mutations were detected prior to the cystoscopical detection, indicating that the urine *FGFR*3 assay can diagnose intravesical recurrence reliably in the postoperative management of NMIBT cases harboring *FGFR3* mutations in primary tumors. As shown in Table 6, the sensitivity of *FGFR*3 mutations and urine cytology in the follow-up samples were mutually exclusive, indicating that the combination of urine *FGFR*3 assay and cytological examinations improved diagnostic sensitivity in detecting tumor relapse. van Rhijn *et al.* reported that the median sensitivity of the cytology for patients under surveillance was 48% and varied between histological grades, ranging from 17% for Grade 1, 34% for Grade 2, and 58% for Grade 3.[24] In the present study, the proportion of tumor grades was 13% (6/45), 78% (35/45), and 9% (4/45) for Grades 1, 2, and 3. Grade 1 and 2 tumors occupied more than 90% of the cohort and the sensitivities of urine cytology in the recurrent cases were 0% (0/9) and 56% (5/9) depending on the presence or absence of the *FGFR*3 mutations.

In conclusion, our results indicate the potential usefulness of the urine *FGFR3* mutation assay as both a significant predictor of intravesical recurrence and a complementary urinary marker for monitoring patients undergoing TURBT in combination with urine cytology. Once *FGFR3* mutations are detected in the primary tumors, the patients should be indicated for monitoring *FGFR3* mutations in the urine sediments, particularly when the tumor cellularity in the preoperative urine sediments is above the cut-off level of 11%.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Cancer Research and for the 3rd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor, and Welfare, Japan. Makito Miyake, MD is the awardee of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research (Japan) for the 3rd Term Comprehensive 10-Year Strategy for Cancer Control (Tokyo, Japan).

Disclosure Statement

None.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Sugano K, Kakizoe T. Genetic alterations in bladder cancer and their clinical applications in molecular tumor staging. *Nat Clin Pract Urol* 2006; **3**: 642–52.
3 Wiener HG, Mian C, Haitel A, Pycha A, Schatzl G, Marberger M. Can urine diagnostic tests replace cystoscopy in the management of bladder cancer? *J Urol* 1998; **159**: 1876–80.
4 Sidransky D, Von Eschenbach A, Tsai YC *et al.* Identification of p53 gene mutations in bladder cancers and urine samples. *Science* 1991; **252**: 706–9.
5 Mao L, Schoenberg MP, Scicchitano M *et al.* Molecular detection of primary bladder cancer by microsatellite analysis. *Science* 1996; **271**: 659–62.
6 Sugano K, Tsutsumi M, Nakashima Y *et al.* Diagnosis of bladder cancer by analysis of the allelic loss of the p53 gene in urine samples using blunt-end single-strand conformation polymorphism. *Int J Cancer* 1997; **74**: 403–6.
7 Shigyo M, Sugano K, Fukayama N *et al.* Allelic loss on chromosome 9 in bladder cancer tissues and urine samples detected by blunt-end single-strand DNA conformation polymorphism. *Int J Cancer* 1998; **78**: 425–9.
8 van Rhijn BW, Lurkin I, Kirkels WJ, van der Kwast TH, Zwarthoff EC. Microsatellite analysis-DNA test in urine competes with cystoscopy in follow-up of superficial bladder carcinoma: a phase II trial. *Cancer* 2001; **92**: 768–75.
9 Shigyo M, Sugano K, Tobisu K, Tsukamoto T, Sekiya T, Kakizoe T. Molecular followup of newly diagnosed bladder cancer using urine samples. *J Urol* 2001; **166**: 1280–5.
10 Steiner G, Schoenberg MP, Linn JF, Mao L, Sidransky D. Detection of bladder cancer recurrence by microsatellite analysis of urine. *Nat Med* 1997; **3**: 621–4.
11 van Rhijn BW, Lurkin I, Chopin DK *et al.* Combined microsatellite and *FGFR3* mutation analysis enables a highly sensitive detection of urothelial cell carcinoma in voided urine. *Clin Cancer Res* 2003; **9**: 257–63.
12 Johnson DE, Williams LT. Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res* 1993; **60**: 1–41.
13 Billerey C, Chopin D, Aubriot-Lorton MH *et al.* Frequent *FGFR3* mutations in papillary non-invasive bladder (pTa) tumors. *Am J Pathol* 2001; **158**: 1955–59.
14 Kimura T, Suzuki H, Ohashi T, Asano K, Kiyota H, Eto Y. The incidence of thanatophoric dysplasia mutations in *FGFR3* gene is higher in low-grade or superficial bladder carcinomas. *Cancer* 2001; **92**: 2555–61.
15 Rieger-Christ KM, Mourtzinos A, Lee PJ *et al.* Identification of Fibroblast Growth Factor Receptor 3 mutations in urine sediment DNA samples complements cytology in bladder tumor detection. *Cancer* 2003; **98**: 737–44.
16 van Oers JM, Lurkin I, van Exsel AJ *et al.* A simple and fast method for the simultaneous detection of nine Fibroblast Growth Factor Receptor 3 mutations in bladder cancer and voided urine. *Clin Cancer Res* 2005; **11**: 7743–8.

17 Miyake M, Sugano K, Kawashima K *et al.* Sensitive detection of *FGFR3* mutations in bladder cancer and urine sediments by peptide nucleic acid-mediated real-time PCR clamping. *Biochem Biophys Res Commun* 2007; **362**: 865–71.
18 Sobin LH, Wittekind C, eds. *TNM Classification of Malignant Tumors*, 6th edn. New York: John Wiley & Sons, 2002.
19 Mostofi FK, Sobin LH, Torloni H. *Histological Typing of Urinary Bladder Tumors: Intenational Classification of Tumors, No. 10.* Geneva: World Health Organization, 1973: 21–31.
20 Knowles MA. Role of *FGFR3* in urothelial cell carcinoma: biomarker and potential therapeutic target. *World J Urol* 2007; **25**: 581–93.
21 van Rhijn BW, Lurkin I, Radvanyi F, Kirkels WJ, van der Kwast TH, Zwarthoff EC. The fibroblast growth factor receptor 3 (*FGFR3*) mutation is a strong indicator of superficial bladder cancer with low recurrence rate. *Cancer Res* 2001; **61**: 1265–8.
22 Hernández S, López-Knowles E, Lloreta J *et al.* Prospective study of *FGFR3* mutations as a prognostic factor in nonmuscle invasive urothelial bladder carcinomas. *J Clin Oncol* 2006; **24**: 3664–71.
23 Burger M, van der Aa MN, van Oers JM *et al.* Prediction of progression of non-muscle-invasive bladder cancer by WHO 1973 and 2004 grading and by *FGFR3* mutation status: a prospective study. *Eur Urol* 2007; **54**: 835–43.
24 van Rhijn BW, van der Poel HG, van der Kwast TH. Urine markers for bladder cancer surveillance: a systematic review. *Eur Urol* 2005; **47**: 736–48.

doi: 10.1111/j.1349-7006.2009.01334.x

Late Recurrence and Progression in Non-muscle–invasive Bladder Cancers After 5-year Tumor-free Periods

Kazuhiro Matsumoto, Eiji Kikuchi, Yutaka Horiguchi, Nobuyuki Tanaka, Akira Miyajima, Ken Nakagawa, Jun Nakashima, and Mototsugu Oya

OBJECTIVES To evaluate the recurrence and progression in patients with non-muscle–invasive bladder tumors who remained tumor-free for at least 5 years, which should assist in the development of schedules of their follow-up evaluations. Non-muscle–invasive bladder tumors that recur or progress at a late time point are not rare.

METHODS Between 1985 and 2002, 814 cases diagnosed with non-muscle–invasive bladder cancer were treated with transurethral resection. Of these 814 cases, 262 patients with no tumor recurrence for more than 5 years were included in the study. The median follow-up interval was 10.0 years.

RESULTS During the follow-up period, 39 tumors (14.9%) showed tumor recurrence. The 5- and 10-year recurrence-free survival rates were 81.6% and 76.0%, respectively. There was no significant difference in tumor recurrence among the low-, intermediate-, and high-risk groups based on the current clinical guideline. Only the use of intravesical mitomycin C was determined to be a significant unfavorable risk factor for late recurrence. Five patients (1.9%) experienced stage progression, 3 of whom did not have metastases at the time of diagnosis of the progression but died because of bladder cancer disease.

CONCLUSIONS After a 5-year tumor-free period, even in the low-risk group, recurrence occurred at a late time point to a degree that was the same as that for the intermediate- and high-risk groups. Finally, some of the high-risk patients experienced late progression with a high degree of malignant behavior, suggesting longer follow-up is needed for each patient. UROLOGY 75: 1385–1391, 2010. © 2010 Elsevier Inc.

Transurethral bladder tumor resection (TUR-BT), intravesical therapy, and frequent follow-up evaluations for non-muscle–invasive bladder cancer are successful at reducing the number of tumor recurrences and at maintaining most bladders free from stage progression. However, some patients with risk factors, such as grade 3 (G3), lamina propria infiltration, multiplicity, a large tumor, and concomitant carcinoma in situ (CIS) are considered to have a lifelong frequency of multiple recurrences and a probability of stage progression.[1-7] These results were obtained from data based on a large number of patients but were examined with median follow-up periods of less than 5 years. Early phase recurrence or progression could be predicted correctly with their data, but they are insufficient to make an accurate estimate of the long-term course of non-muscle–invasive bladder cancer because of the short follow-up in their current analyses. Long-term follow-up data would provide details of the exact outcome of late recurrence and progression in non-muscle–invasive bladder cancer.

The recommended follow-up schedules proposed by the published guidelines vary, especially after 5-year tumor-free periods.[8-11] Fujii et al[12] pointed out that tumor recurrence and stage progression continue to occur in patients with superficial bladder cancer who remained tumor-free for more than 4 years after initial treatment. Meanwhile, Leblanc[13] reported that even in Ta G1 tumors a significant number of recurrences (14%) were observed more than 5 years after the first tumor. In contrast, Haukaas et al[14] stated that routine cystoscopy can possibly be discontinued in patients with low-grade, low-stage disease in the absence of recurrences during follow-up. Mariappan et al[15] also proposed that patients with Ta G1 tumors could be discharged with a 5-year tumor-free period on the basis of their long-term follow-up data.

In this study, we reviewed 262 patients with diagnosed non-muscle–invasive bladder cancer who remained tumor-free for at least 5 years, to analyze their tumor recurrence and stage progression at a late time point.

From the Department of Urology, Keio University School of Medicine, Tokyo, Japan; and Department of Urology, Tokyo Medical University, Tokyo, Japan

Reprint requests: Eiji Kikuchi, M.D., Department of Urology, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: eiji-k@kb3.so, http://net.ne.jp

Submitted: March 8, 2009, accepted (with revisions): September 25, 2009

0090-4295/10/$34.00
doi:10.1016/j.urology.2009.09.088

Table 1. Univariate and multivariate analyses on tumor recurrence after 5-year tumor-free periods

		Univariate	Multivariate	
	No. of Patients	*P*	Risk Ratio (95% CI)	*P*
Recurrence history		.328		
Primary	188			
Recurrence	74			
Pathologic stage		.067		
pTa	196			
pT1	66			
Tumor grade		.271		
G1-2	191			
G3	71			
Multiplicity		.156		
Solitary	149			
Multiple	113			
Carcinoma in situ		.556		
Present	18			
Not present	244			
Adjuvant BCG treatment		.230		
Performed	176			
Not performed	86			
Adjuvant MMC treatment		<.001		.001
Performed	27		4.00 (1.98-7.79)	
Not performed	235		1.00	

CIS indicates carcinoma in situ; BCG = bacillus Calmette–Guérin; MMC = mitomycin C.

These data should assist with the development of schedules for their follow-up evaluations beyond 5 years.

MATERIAL AND METHODS

The medical records of our institute between 1985 and 2002 were reviewed, and 814 patients with diagnosed non-muscle–invasive bladder cancer (confined to the epithelial mucosa or lamina propria infiltration) who were treated with TUR-BT at our university hospital were identified. Of these 814 cases, 262 (32.2%) patients who suffered no recurrence of the tumor for more than 5 years after TUR-BT were included in the present study. Their mean age was 61.6 years (range, 22.0-84.1 years) and the median follow-up interval was 10.0 years (range, 5.1-24.5 years).

These cases were routinely assessed by urine cytology and cystoscopy every 3 months for the first 2 years after TUR-BT, every 6 months for the next 3 years, and every 6-12 months thereafter. Intravenous urography, ultrasonography, and/or computed tomography (CT) were used to evaluate the bladder and upper urinary tract every year for 5 years after the initial treatment and then every 1 or 2 years thereafter. Bacillus Calmette–Guérin (BCG) treatment was introduced at our hospital in 1990, whereas others with high-risk tumors were treated with intravesical instillation of mitomycin C (MMC). Whether intravesical therapies should be performed depends on the attending doctors' preference. BCG treatment (Tokyo, 172 strain) at a dose of 80 mg or MMC at a dose of 30 mg was begun 4-5 weeks after TUR-BT, and continued weekly for 6-8 weeks.

The starting point of the study was 5 years after TUR-BT treatment. The primary endpoint was the time to tumor recurrence, and the secondary endpoint was the time to stage progression. Recurrence was defined as a new tumor appearing in the bladder after initial clearance. Progression was defined as confirmed histologic muscle invasion (pathologic stage T2 or higher disease) or detectable distant metastases.

The characteristics of the recurrence history, tumor grade, initial number of tumors, pathologic stage, and the presence of CIS were determined and then the patients were stratified into 3 risk groups, referring to the current published guideline of the American Urological Association.[11] We constructed this risk stratification by interpreting low grade as G1-2 and high grade as G3. Tumor size was excluded in the analysis because some of the data were not available.

Recurrence-free and progression-free survival curves were constructed using the Kaplan–Meier method and were compared with the log-lank test. Differences among groups were regarded as significant when $P < .05$. Univariate and multivariate analyses were done, using the Cox proportional hazards model with stepwise forward selection, to determine risk factors of recurrence.

RESULTS

The clinical characteristics of the patients who were free from tumor recurrence 5 years after TUR-BT treatment are listed in Table 1. G3 tumors were found in 71 patients, consisting of 35 Ta G3 and 36 T1 G3 tumors. BCG intravesical immunotherapy was performed in 176 patients (67.2%), whereas 27 patients (10.3%) received MMC instillation. After 5-year tumor-free periods, 39 tumors had recurred and 5 tumors had progressed. Using the Kaplan–Meier method, the 5-, 10-, and 15-year recurrence-free survival rates were 81.6%, 76.0%, and 68.5%, and the 5-, 10-, and 15-year progression-free survival rates were 97.5%, 97.5%, and 97.5%, respectively. Upper urinary tract recurrence (UTR) was observed in only 1 patient (0.4%) in the follow-up period.

Next, we divided all patients into 3 risk groups, referring to the current guideline statements in the American Urological Association guideline (Table 2),[11] and then

Table 2. Risk stratification

	Definition	No. of Patients	No. of Patients Treated With BCG and MMC
Low-risk group	Solitary Ta G1-2	80 Patients (30.5%)	BCG 30 patients MMC 13 patients
Intermediate-risk group	Multiple Ta G1-2 Recurrent Ta G1-2	79 Patients (30.2%)	BCG 57 patients MMC 8 patients
High-risk group	Ta G3 T1 G2-3 Concomitant CIS	103 Patients (39.3%)	BCG 89 patients MMC 6 patients

constructed Kaplan–Meier curves of the recurrence-free survival rates in the low-, intermediate-, and high-risk groups. There were no significant differences among the 3 risk groups (Fig. 1A). However, none of the 37 low-risk patients with 10-year tumor-free periods experienced tumor recurrence, and the median follow-up interval was 14.0 years. Meanwhile, tumor recurrences were observed in 4 of 26 intermediate-risk patients (15.4%) and 1 of 36 high-risk patients (2.8%) after 10-year tumor-free periods. Most of our cases underwent an induction course of BCG and Kaplan–Meier curves were constructed for these 176 patients. Again there was no significant difference among the 3 risk groups with respect to tumor recurrence (Fig. 1B).

Next, we performed univariate and multivariate analyses to determine the risk factors for late tumor recurrence. Among the clinicopathological variables examined, only the use of intravesical MMC was determined to be a significant unfavorable risk factor for late recurrence (Table 1). The significant differences in recurrence-free survival rates among patients treated with MMC, BCG, and no adjuvant intravesical therapy are seen in Figure 1C.

Five patients experienced stage progression, consisting of 2 T1 G3 cases, 2 T1 G2 cases, and 1 case of a Ta G3 tumor with concomitant CIS, as summarized in Table 3. These 5 patients all belonged to the high-risk group. Radical cystectomy was performed in 2 patients, but they subsequently died because of bladder cancer. Two patients underwent radiation therapy, 1 of whom died of bladder cancer. The remaining patient was treated with a combination of radiation and systemic chemotherapy, and she was diagnosed with lymph node metastases at the last follow-up. Another high-risk group patient with solitary Ta G3 tumors experienced UTR later, but she was cured by a nephroureterectomy.

COMMENT

In the present study, we described the rates of recurrence and progression in 262 patients remaining tumor-free for 5 years after TUR-BT. We believe that these data provide a more accurate description of the biological behavior of non-muscle–invasive bladder cancer at a late time point. We divided the patients on the basis of their pathologic factors and demonstrated that this risk stratification cannot predict their recurrence at a late time point. Important findings were that tumors in the low-risk group (solitary Ta G1-2) proved to have the potential to recur at a point beyond 5 years, which is similar to that for the high-(Ta G3, T1 G2-3, and/or concomitant CIS) and intermediate-risk groups (multiple and/or recurrent Ta G1-2), and that stage progression and UTR were observed in the high-risk group even after a 5-year tumor-free period, suggesting the importance of long-term follow-up in each patient even after a 5-year tumor dormancy period.

Bladder tumors that recur or progress at a late time point are not rare, and so long-term follow-up data are very important to ascertain the accurate biological outcome of non-muscle–invasive bladder cancer. Several studies have analyzed the outcome of long-term follow-up of non-muscle–invasive bladder cancer. Cookson et al[16] evaluated 86 patients with high-risk superficial bladder cancers followed up for a minimum of 15 years and reported that 29 patients (33.7%) died of the disease. In their series, 70 patients (81.4%) had CIS lesions, a percentage which was much higher than that seen in current clinical practice. Holmäng et al[17] reported that 39 of 176 patients (22.2%) with Ta or T1 bladder cancer had died of the disease after at least 20 years follow-up. Their data did not include subjects treated with BCG therapy. Haukaas et al[14] reported 217 cases of non-muscle–invasive bladder cancer with a mean follow-up period of 9 years, and again BCG adjuvant therapy was not performed. The follow-up protocol in the present study was thought to be suitable in the current clinical setting in the BCG era. This study was based on data from a single institution in Japan, which enabled us to obtain objective data with little bias or loss to follow-up.

Some previous reports have stated that low-grade, low-stage disease in the absence of recurrences during follow-up have a low risk of subsequent recurrence.[14,15] However, Mariappan et al[18] reanalyzed the data and stated later that the more contemporary patients had a greater risk of delayed first recurrence beyond 5 years. Our data also demonstrated that recurrence continues to occur even after 5-year tumor-free periods in every risk group.

In our series of selected patients, the major well-known risk factors were not shown to be statistically significant

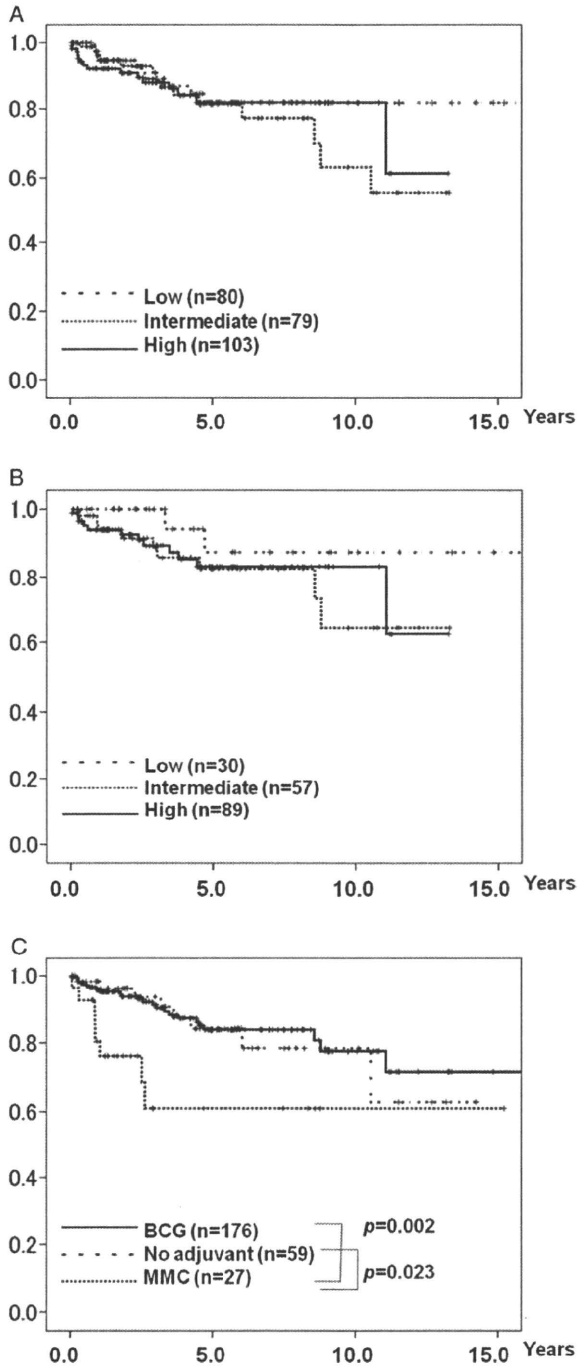

Figure 1. Differences in recurrence-free survival rates **(A)** among the risk groups in overall patient population (262 patients); **(B)** among the risk groups in 176 patients who received an induction course of Bacillus Calmette–Guérin; **(C)** among the patients treated with mitomycin C, Bacillus Calmette–Guérin, and no adjuvant intravesical therapy. The time point 0 was 5 years after initial transurethral bladder tumor resection.

for late recurrences (Table 1). This is probably because tumors with those factors have a higher risk of recurrence at an early time point and were not included in this study, and we believe that these factors are useful for predicting early phase recurrence. An induction course of BCG instillation appears, based on our data, to not prevent late tumor recurrence. However, no patients in this study received maintenance BCG immunotherapy, which may provide long-term benefits as Lamm et al[19] reported. Meanwhile, the current patients who received an induction course of intravesical MMC therapy had a higher recurrence rate after a 5-year tumor-free period (Fig. 1C). This finding is consistent with a previous study in which intravesical chemotherapy failed when followed for a longer period.[20] Fujii et al[12] analyzed 100 patients with superficial bladder cancer who remained tumor-free for more than 4 years, and they also reported that only the use of intravesical chemotherapy was determined to be a significant unfavorable risk factor for late recurrence. Of our study population who had a 5-year tumor-free period after TUR-BT, a small number of patients (27, 10.3%) received an induction course of intravesical MMC instillation, and no patients received maintenance chemotherapy. Therefore, the high degree of selection bias may have led to the results. However, we believe that intravesical chemotherapy might reduce the recurrence rate in the short term, while failing to decrease the incidence of recurrence when followed for longer periods. Hinotsu et al[21] have clearly demonstrated that the risk of tumor recurrence after 500 days post-TUR-BT is not reduced by intravesical chemotherapy. Consequently, we think that intravesical chemotherapy might delay the tumor recurrence, and thus patients who received an induction course of intravesical MMC instillation might have a risk for late tumor recurrence and should be followed up carefully.

Only a few reports have investigated the possibility of developing stage progression after a long tumor-free period, and the incidence varied from 1.7% to 5.0%.[12,17] In the present study, 5 patients (1.9%) experienced stage progression and another patient had UTR later on. All of these 6 patients belonged to our high-risk group, and no patients in the low- or intermediate-risk groups experienced stage progression or UTR during our follow-up period. Three of the 5 patients who had stage progression after a 5-year tumor free period did not have metastases at the time of diagnosis of the progression but did die of bladder cancer disease, even though the cancer progression was treated aggressively. This may indicate that stage progression at a point after a 5-year tumor free period exhibits highly malignant behavior and patients in the high-risk group should be followed up closely well beyond 5 years.

No guideline outlines a specific follow-up schedule for low-risk patients after a 5-year tumor-free period. We believe that, even for patients in our low-risk group (solitary Ta G1-2), follow-up evaluation at least once a year after a 5-year tumor-free period should be performed because the probability of recurrence is equal to that of the intermediate- and high-risk groups and should be continued for at least 10 years. In our patients, none of

Table 3. Clinicopathological profiles of 5 patients with stage progression

Histology of TUR-BT	Adjuvant Intravesical Therapy	Years at Diagnosis Progressed	Stage at Diagnosis Progressed	Treatment for Progression	Outcome	Years at Last Follow-Up
Multiple T1 G2	None	5.4	T2N0M0	Radiation	DOC	6.0
Multiple T1 G3	MMC	5.5	T3bN1M0	RC	DOD	6.5
Solitary T1 G3	BCG	6.4	T3N0M0	Radiation and chemotherapy	AWD	10.2
Solitary T1 G2	BCG	6.8	T3N0M0	Radiation	DOD	8.0
Multiple Ta G3 with CIS	BCG	8.5	T3aN0M0	RC	DOD	11.0

RC indicates radical cystectomy; DOC = dead on other causes; DOD = dead on disease; AWD = alive with disease.

the low-risk tumors, which consisted of 8 solitary Ta G1 tumors and 29 solitary Ta G2 tumors, with a 10-year tumor-free period experienced recurrence later. Cytology is known to be a convenient noninvasive test for screening of high-risk population. However, clinical experience has shown some limitations of urine cytology in low-grade tumors, in which the sensitivity is low.[22] In the present study, none of our 80 low-risk patients experienced stage progression or UTR after a 5-year tumor-free period. Thirteen patients experienced late tumor recurrence in the low-risk group, only 1 (7.7%) of whom had G3 components. During the follow-up period, no positive urine cytology result was observed in this low-risk group. Therefore, we believe routine cytology examination may not be necessary in their later follow-up due to the low possibility of high-grade tumor appearance in the low-risk group. In addition, no low-risk patients in our study experienced UTR after a 5-year tumor-free period. Hurle et al[23] found that the incidence of UTR was very low (0.9%) in low-risk patients. Wright et al[24] also reported that of 99 338 patients with bladder cancer, UTR developed in only 0.8% and most patients with low-grade tumors not involving the ureteral orifice have a sufficiently low risk of UTR. Thus, the costs and the demerits of radiation exposure from radiographic imaging such as CT for frequent surveillance may outweigh the benefits of early detection of the very few UTR in low-risk patients.

Meanwhile, for high-risk patients, annual cystoscopy and imaging of the upper urinary tract are recommended in the European Association of Urology guideline after a 5-year tumor-free period.[9] The National Comprehensive Cancer Network guideline also stated that intermediate- and high-risk patients should receive annual cystoscopy, while imaging of the upper urinary tract should be performed every 1 or 2 years after 5 years follow-up.[10] In our study, the high-risk patients (Ta G3, T1 G2-3, or concomitant CIS) with a 5-year tumor-free period may still experience progression and UTR, in addition to recurrence. We recommend intense follow-up evaluations for these patients, including CT and CT urography which can detect both progression disease and UTR accurately,[25] and that they should be performed after 5-year tumor-free periods because of the poor prognosis of our progression cases.

This study has several limitations. First, it was performed in a retrospective manner and in a limited number of patients. Disparities in intravesical therapies among the risk groups may have introduced bias into the results. Not all patients received 1 immediate postoperative installation of chemotherapy. However, the rationale for 1 immediate postoperative installation of chemotherapy seems to include the destruction of residual microscopic tumors at the site of TUR-BT and of circulating cells that could be implanted. It has been reported that the therapeutic benefit of the positive effect of a single immediate chemotherapy instillation was limited to early recurrence, mainly during the first 2 years, and not maintained with long-term follow-up.[26] Therefore, we believe that the 1 immediate postoperative installation of chemotherapy in the present study may not have affected the late tumor recurrence after 5-year tumor-free periods. Meanwhile, the present study has also another limitation that no patients received second-look TUR or any maintenance intravesical therapies, which may have improved the results. Further study aimed at evaluating the importance of follow-up for a long-term in these therapeutic situations is warranted.

CONCLUSIONS

Our long-term follow-up data indicated that even in the low-risk group, recurrence occurred at a late time point, an observation that is the same as for the intermediate- and high-risk groups. Also, some of the high-risk patients experienced late progression or UTR with poor survival even after a 5-year tumor-free period, suggesting the need for longer follow-up in all patients.

References

1. Kiemeney LA, Witjes JA, Heijbroek RP, et al. Predictability of recurrent and progressive disease in individual patients with primary superficial bladder. *J Urol.* 1993;150:60-64.
2. Kurth KH, Denis L, Bouffioux C, et al. Factors affecting recurrence and progression in superficial bladder tumours. *Eur J Cancer.* 1995; 31A:1840-1846.
3. Millan-Rodriguez F, Chechile-Toniolo G, Salvador-Bayarri J, et al. Multivariate analysis of the prognostic factors of primary superficial bladder cancer. *J Urol.* 2000;163:73-78.
4. Millan-Rodriguez F, Chechile-Toniolo G, Salvador-Bayarri J, et al. Primary superficial bladder cancer risk groups according to progression, mortality and recurrence. *J Urol.* 2000;164:680-684.
5. Ali-El-Dein B, Sarhan O, Hinev A, et al. Superficial bladder tumours: analysis of prognostic factors and construction of a predictive index. *BJU Int.* 2003;92:393-399.

6. Sylvester RJ, van der Meijden AP, Oosterlinck W, et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. *Eur Urol.* 2006;49:466-477.
7. Kikuchi E, Fujimoto H, Mizutani Y, et al. Clinical outcome of tumor recurrence for Ta, T1 non-muscle invasive bladder cancer from the data on registered bladder cancer patients in Japan: 1999-2001 report from the Japanese Urological Association. *Int J Urol.* 2009;16:279-286.
8. Persad R, Lamm D, Brausi M, et al. Current approaches to the management of non-muscle invasive bladder cancer: comparison of current guidelines and recommendations. *Eur Urol Suppl.* 2008;7: 637-650.
9. Bajuk M, Oosterlinck W, Sylvester RJ, et al. European Association Of Urology Guidelines On Ta T1 (Non-Muscle Invasive) Bladder Cancer (Update March 2008). Arnhem, the Netherlands: European Association of Urology; 2008.
10. National Comprehensive Cancer Network. Clinical Practice Guidelines in Oncology: Bladder Cancer Including Upper Tract Tumors and Urothelial Carcinoma of the Prostate. Jenkintown, PA: NCCN; 2009.
11. American Urological Association. Guidelines for the Management of Non-Muscle Invasive Bladder Cancer (Stages Ta, Tl, and Tis) (2007 Update). Linthicum, MD: AUA; 2007.
12. Fujii Y, Fukui I, Kihara K, et al. Late recurrence and progression after a long tumor-free period in primary Ta and T1 bladder cancer. *Eur Urol.* 1999;36:309-313.
13. Leblanc B, Duclos AJ, Benard F, et al. Long-term follow-up of initial Ta grade 1 transitional cell carcinoma of the bladder. *J Urol.* 1999;162:1946-1950.
14. Haukaas S, Daehlin L, Maartmann-Moe H, et al. The long-term outcome in patients with superficial transitional cell carcinoma of the bladder: a single-institutional experience. *BJU Int.* 1999;83: 957-963.
15. Mariappan P, Smith G. A surveillance schedule for G1Ta bladder cancer allowing efficient use of check cystoscopy and safe discharge at 5 years based on a 25-year prospective database. *J Urol.* 2005; 173:1108-1111.
16. Cookson MS, Herr HW, Zhang ZF, et al. The treated natural history of high risk superficial bladder cancer: 15-year outcome. *J Urol.* 1997;158:62-67.
17. Holmäng S, Hedelin H, Anderström C, et al. The relationship among multiple recurrences, progression and prognosis of patients with stages Ta and T1 transitional cell cancer of the bladder followed for at least 20 years. *J Urol.* 1995;153:1823-1826.
18. Mariappan P, Smith G, Alastair D, et al. Pattern of recurrence changes in noninvasive bladder tumors observed during 2 decades. *J Urol.* 2006;177:867-875.
19. Lamm DL, Blumenstein BA, Crissman JD, et al. Maintenance bacillus Calmette-Guerin immunotherapy for recurrent Ta, T1 and carcinoma in situ transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study. *J Urol.* 2000;163: 1124-1129.
20. Lamm DL, Riggs DR, Traynelis CL, et al. Apparent failure of current intravesical chemotherapy prophylaxis to influence the long-term course of superficial transitional cell carcinoma of the bladder. *J Urol.* 1995;153:1444-1450.
21. Hinotsu S, Akaza H, Ohashi Y, et al. Intravesical chemotherapy for maximum prophylaxis of new early phase superficial bladder carcinoma treated by transurethral resection. *Cancer.* 1999;86: 1818-1826.
22. Bastacky S, Ibrahim S, Wilczynski SP, et al. The accuracy of urinary cytology in daily practice. *Cancer.* 1999;87:118-128.
23. Hurle R, Losa A, Manzetti A, et al. Upper urinary tract tumors developing after treatment of superficial bladder cancer: 7-year follow-up of 591 consecutive patients. *Urology.* 1999;53:1144-1148.
24. Wright JL, Hotaling J, Porter MP. Predictors of upper tract urothelial cell carcinoma after primary bladder cancer: a population based analysis. *J Urol.* 2009;181:1035-1039.
25. Zhang J, Gerst S, Lefkowitz RA, et al. Imaging of bladder cancer. *Radiol Clin North Am.* 2007;45:183-205.
26. Solsona E, Iborra I, Ricós JV, et al. Effectiveness of a single immediate mitomycin C instillation in patients with low risk superficial bladder cancer: short and long-term follow-up. *J Urol.* 1999;161:1120-1123.

EDITORIAL COMMENT

The urologic literature is rich in retrospective studies of clinical outcomes for noninvasive bladder cancer treated with TUR-BT (either alone or with adjuvant intravesical therapy) with short- and intermediate-term results. Based on these studies, a need to better categorize patients with TaT1 tumors arose, and patients were divided into risk groups (low-risk, intermediate-risk, and high risk) based on prognostic factors derived from multivariate analyses. When using these risk groups, however, no difference is usually made between the risk of recurrence and progression. Although prognostic factors may indicate a high risk of recurrence, the risk of progression may still be low and other tumors may have a high risk of both recurrence and progression. In contrast, this type of risk classification is not adapted to predict recurrence or progression at a late time point.

In this study, 262 (32.2%) of 814 patients with noninvasive bladder cancer, managed between 1985 and 2002, did not have a tumor recurrence for >5 years and were followed up for a median of 10 years. The starting point was 5 years after TUR-BT treatment. Patients received either BCG or MMC, 4-5 weeks after resection and continued for 6-8 weeks. Intravesical BCG immunotherapy was performed in 67% patients, while 10% received MMC. After a 5-year tumor-free period, 39 (15%) tumors recurred and 5 (1.9%) tumors progressed. Only the use of intravesical mitomycin C was determined to be a significant unfavorable risk factor for late recurrence.

Although this study has some limitations, which have been mentioned by the authors, it still leaves us some messages. First, the risk classification cannot predict recurrence or progression at long term. The use of a scoring systems and risk tables[1,2] might be a better option, as demonstrated by the European Organization for Research and Treatment of Cancer (EORTC). The scoring system is based on the 6 most significant clinical and pathologic factors, which are as follows: number of tumors, tumor size, prior recurrence rate, T category, presence of concomitant CIS, and tumor grade.[1] These scoring systems give a risk stratification model to provide accurate estimates of recurrence and progression probability after intravesical adjuvant therapy.[1,2] Second, long-term follow-up (>5 years) for patients with noninvasive bladder cancer who have had a 5-year tumor-free period should be recommended. This follow-up should be done using cystoscopy alone (at increasing intervals) for low-risk patients, cytology and cystoscopy for intermediate- and high-risk patients, and upper tract imaging studies should be done for high-risk patients. Third and last, a big question arises: will the immediate (<24 hours post-TUR-BT) intravesical chemotherapy treatment, which has proved to prevent short-term recurrence (in the first 2 years), have an effect on longer-term follow-up because of a delayed time point for recurrence of patients with TaT1 bladder tumors receiving this type of therapy. Only studies with longer-term outcomes (>5 years) that include patients with the contemporary management of TaT1 bladder tumors will respond to this question.

 DOI 10.1002/eji.201040773 Eur. J. Immunol. 2011. 41: 246–251

European Journal of Immunology SHORT COMMUNICATION

IL-17 production by γδ T cells is important for the antitumor effect of *Mycobacterium bovis* bacillus Calmette-Guérin treatment against bladder cancer

Ario Takeuchi[1,2], Takashi Dejima[1,2], Hisakata Yamada[1], Kensuke Shibata[1], Risa Nakamura[1], Masatoshi Eto[3], Tatsuya Nakatani[4], Seiji Naito[2] and Yasunobu Yoshikai[1]

[1] Division of Host Defense, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan
[2] Department of Urology, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan
[3] Department of Urology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan
[4] Department of Urology, Osaka City University Graduate School of Medicine, Osaka, Japan

Intravesical inoculation of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) has been used for the treatment of bladder cancer. Recent studies implied the requirement of neutrophil infiltration for the antitumor effect. In this study, we found that IL-17 was produced in the bladder after BCG treatment, preceding the infiltration of neutrophils. Neutrophils in the bladder after BCG treatment were reduced in IL-17-deficient mice, in which BCG-induced antitumor effect against intravesically inoculated bladder cancer was abolished. Notably, the level of IL-17 production and the number of neutrophils in BCG-treated bladder was reduced in γδ T-cell-deficient mice but not in CD4-depleted mice. Survival of bladder cancer-inoculated γδ T-cell-deficient mice was not improved by BCG treatment. These results suggest that IL-17-producing γδ T cells play a key role in the BCG-induced recruitment of neutrophils to the bladder, which is essential for the antitumor activity against bladder cancer.

Key words: γδ T cells · Bladder tumor · BCG · IL-17 · Neutrophils


Supporting Information available online

Introduction

In 1976, Morales *et al.* reported intravesical inoculation of *Mycobacterium bovis* BCG as an effective adjuvant therapy for bladder cancers [1]. Thereafter, intravesical immunotherapy with BCG has been used for 30 years, however the antitumor effector mechanisms remain elusive. Recent studies demonstrated that neutrophils infiltrated in the bladder after BCG treatment played a key role in the antitumor effect [2]. Expression of TRAIL on neutrophils in voided urine following BCG therapy suggests a direct antitumor effect of neutrophils [3, 4]. In addition, neutrophils isolated from BCG-treated bladder produced CC (*e.g.* MIP-1α) as well as CXC chemokines (*e.g.* IL-8 and GRO-α). The chemokines released by activated neutrophils attract monocytes, which in turn result in BCG-induced CD4 T-cell-migration [2]. Th1-polarized cell-mediated immunity, which includes NK cells, and $CD8^+$ and $CD4^+$ T cells, was also involved in the antitumor effect of BCG immunotherapy [5–7]. Thus, neutrophils

Correspondence: Dr. Hisakata Yamada
e-mail: hisakata@bioreg.kyushu-u.ac.jp

