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BJUI
BJU INTERNATIONAL

Expression of integrin proteins in non-muscle-invasive bladder cancer: significance of intravesical recurrence after transurethral resection

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Study Type – Aetiology (case control)
Level of Evidence 3b

What's known on the subject? and What does the study add?
A number of studies have reported several clinicopathological factors closely associated with intravesical recurrence of non-muscle invasive bladder cancer (NMIBC). In addition, various types of molecular markers have been shown to be useful for predicting intravesical recurrence of NMIBC following transurethral resection (TUR).

Of six subunits of integrin proteins, including α2, α3, α5, α6, β1 and β4, the expression level of the β4 subunit in NMIBC, in addition to pathological T stage and concomitant carcinoma *in situ* appeared to be independently related to intravesical recurrence. Therefore, consideration of the expression levels of integrins, particularly that of the β4 subunit, in TUR specimens would contribute to further accurate prediction of intravesical recurrence of NMIBC.

OBJECTIVES

- To evaluate the expression of integrin proteins, a family of transmembrane heterodimers, in non-muscle-invasive bladder cancer (NMIBC).
- To assess the significance of these proteins as prognostic indicators in patients undergoing transurethral resection (TUR).

PATIENTS AND METHODS

- The present study comprised 161 patients diagnosed as having NMIBC after TUR.
- Expression levels of six subunits of integrin proteins, including α2, α3, α5, α6, β1 and β4, were measured in TUR specimens by immunohistochemical staining.

RESULTS

- Of the six proteins, expression levels of α2-, α3-, α6- and β4-subunits were significantly associated with the incidence of intravesical recurrence. Univariate analysis identified expression levels of α3-, α6- and β4-subunits as important predictors of intravesical recurrence, while tumour size, pathological T stage and concomitant carcinoma *in situ* (CIS) were also important.
- Multivariate analysis showed that the expression level of the β4 subunit, pathological T stage and concomitant CIS are independently related to intravesical recurrence.
- There were significant differences in intravesical recurrence-free survival for patients who were positive for the three independent risk factors; intravesical recurrence occurred in 10 of 49 (20.4%) patients who were negative for all risk factors, 31 of 68 who were positive for one risk factor (45.6%), and 30 of 44 who were positive for two or three risk factors (68.2%).

CONCLUSIONS

- Consideration of the expression levels of integrins, particularly those of the β4 subunit, in TUR specimens, in addition to conventional variables, would contribute to accurate prediction of intravesical recurrence after TUR for NMIBC patients.

KEYWORDS

non-muscle-invasive bladder cancer, transurethral resection, intravesical recurrence, integrin

INTRODUCTION

Approximately 80% of newly diagnosed bladder cancers are non-muscle- invasive tumours that are limited to the urothelium or infiltrate no deeper than the lamina propia. Complete transurethral resection (TUR) of the visible tumour burden, which is the current standard of care for patients with non-muscle-invasive bladder cancer (NMIBC), generally achieves a favourable prognosis with 5-year survival rates >80% [1]. However, intravesical recurrence after TUR has been reported in 30 to 80% of patients with NMIBC [1–3]. Intensive efforts, therefore, have been paid to the development of systems that can accurately predict the outcome of TUR, and these systems are of great value for planning postoperative adjuvant therapy, as well as for follow-up schedules for individual patients with NMIBC.

To date, a number of studies have reported risk factors closely associated with intravesical recurrence of NMIBC, including tumour multiplicity, maximal tumour size, grade, stage, growth pattern and microvascular invasion [3–7]; however, these outcomes are not consistent. Hence, in order to provide more reliable information regarding the probability of intravesical recurrence in patients with NMIBC after TUR, several investigators have evaluated the value of various types of molecular markers, and some of these molecules were shown to be significantly related to postoperative prognostic outcomes [8–18]. For example, Mhawech-Fauceglia *et al.* [9] identified fibroblast growth factor receptor-3 as an important factor in predicting the time to recurrence in NMIBC using a high-throughput tissue microarray. Miyake *et al.* [14] and Behnsawy *et al.* [18] also reported that strong clusterin and weak p21 expression in resected NMIBC specimens could be important predictive factors of intravesical recurrence in patients with NMIBC after TUR. However, no systems have been introduced into clinical practice for predicting outcomes in patients with NMIBC who were treated with TUR.

Integrins, a family of transmembrane heterodimers, each of which is composed of a single α- and β-subunit, function as receptors for extracellular matrix molecules and mediate signal transduction for the control of diverse cell functions, such as survival, proliferation, differentiation, angiogenesis and migration [19,20]. Furthermore, aberrant expression of integrin has been shown to play an important role in the acquisition of aggressive phenotype in cancer cells [21,22]. Few studies have, however, investigated the role of integrins in bladder cancer progression [23–26]. It is, therefore, not known whether integrins have a marked impact on the prognosis of patients with bladder cancer, and with NMIBC in particular. Considering this, we evaluated the expression levels of six subunits of integrin proteins, including α2, α3, α5, α6, β1 and β4, in TUR specimens using immunohistochemical staining, and analysed these outcomes according to several clinicopathological variables.

PATIENTS AND METHODS

The present study comprised 161 consecutive patients who were treated with TUR for newly diagnosed primary bladder cancer between January 2000 and December 2007 at Kobe University Hospital, and who were subsequently diagnosed as having NMIBC (i.e. stage Ta or T1 tumour). Informed consent was obtained from each patient, and the study design was approved by our institution's Research Ethics Committee. Growth pattern was macroscopically classified into either the papillary or non-papillary type. Tumour size was defined as the largest tumour measured using a 1-cm long resection loop. Complete resection of all visible tumours was done, and several deep muscular samples were obtained with the resection instrument. Irrespective of findings from preoperative urinary cytology, random bladder biopsy that targeted the trigone, posterior wall, bilateral lateral walls, dome, anterior wall, prostatic urethra in men and/or suspicious regions, was performed before TUR for all patients. Pathological examinations were carried out by a single pathologist according to the 2002 American Joint Committee on Cancer TNM classification system.

As a rule, the indication for adjuvant intravesical instillation therapy was defined as the presence of concomitant cancer *in situ* (CIS) and/or T1G3 disease. BCG was, therefore, administered for the majority of patients who received adjuvant intravesical instillation therapy in the present series. Follow-up of patients after TUR of NMIBC was carried out based on the relatively intensive schedule previously described [18], i.e. cystoscopy and urinary cytological examination were performed every 3 months for 3 years after TUR, and then every 6 months until 5 years after TUR, and intravenous pyelography was performed every 6 months until 3 years after TUR and then annually until 5 years after TUR. On detection of tumours or hyperemic mucosa by cystoscopy and/or positive findings from urinary cytology, transurethral biopsy of the abnormal region and/or TUR of the tumour were performed.

Immunohistochemical staining of TUR specimens was performed as previously described [27]. Briefly, sections from formaldehyde-fixed, paraffin-embedded tissue from 161 specimens were deparaffinized by xylene and rehydrated in decreasing concentrations of ethanol. After blocking endogenous peroxidase with 3% hydrogen peroxidase in methanol, sections were boiled on 0.01 M citrate buffer for 10 min and incubated with 5% normal blocking serum in tris-buffered saline for 20 min. The sections were then incubated with the following antibodies: anti-human α2 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human α3 mouse monoclonal antibody (Santa Cruz Biotechnology), anti-human α5 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-human α6 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-human β1 rabbit monoclonal antibody (Epitomics, Burlingame, CA, USA) and anti-human β4 rabbit polyclonal antibody (Santa Cruz Biotechnology). The sections were then incubated with biotinylated goat anti-mouse or rabbit IgG (Vector Laboratories, Burlingame, CA, USA). After incubation in avidin–biotin peroxidase complex for 30 min, the samples were exposed to diaminobenzidine tetrahydrochloride solution and counterstained with methyl green (Vector Laboratories).

Staining results were interpreted by two independent observers who were blinded to the clinicopathological data. If discordant interpretations were obtained, differences were resolved by joint review and/or consultation with a third observer familiar with immunohistochemical pathology. In this series, staining of each protein was characterized based on five staining levels (0, no staining; 1, 1% to 25% of cells showing membrane bound expression; 2, 25% to 50%; 3, 50% to 75%; and 4, 75% to 100%). For the purposes of presentation, the staining result was considered strong if more than 50% of cells expressed the antigen (staining levels 3 and 4) and weak if 50% or fewer of the cells showed a staining signal (levels 0 to 2) [28].

All statistical analyses were performed using Statview 5.0 software (Abacus Concepts, Inc., Berkeley, CA). The chi-squared test was used to analyse associations between intravesical recurrence and several variables. Intravesical recurrence-free survival rates were calculated by the Kaplan–Meier method, and differences were determined by log-rank test. The prognostic significance of certain factors was assessed by the Cox proportional hazards regression model. $P < 0.05$ was considered significant.

RESULTS

During the median observation period of 47 months (range, 13–93), intravesical disease

TABLE 1 Characteristics of 161 patients with MIBC who underwent TUR with regard to intravesical recurrence

	Intravesical recurrence			Intravesical recurrence-free survival	
Variables	Negative ($N = 90$)	Positive ($N = 71$)	*P* value	3-year recurrence-free survival rate (%)	*P* value
Age (years)			0.71		0.94
<70	43	36		54.6	
	47	35		53.0	
Gender			0.28		0.26
M	79	58		53.9	
F	11	13		53.9	
Preoperative urinary cytology			0.97		0.79
Negative	53	42		53.8	
Positive	37	29		54.4	
Multiplicity of tumour			0.35		0.23
Single	46	31		59.5	
Multiple	44	40		48.3	
Maximal tumour size			<0.001		0.0011
≤3 cm	72	37		63.2	
>3 cm	18	34		36.8	
Tumour type			0.13		0.068
Papillary	80	57		57.6	
Non-papillary	10	14		34.7	
T stage			0.081		0.022
Ta	65	42		60.6	
T1	25	29		40.6	
Grade			0.37		0.37
G1	29	20		57.6	
G2	49	40		54.7	
G3	12	11		42.8	
CIS			0.0038		0.0010
Negative	76	46		61.6	
Positive	14	25		31.6	
Adjuvant intravesical instillation therapy			0.12		0.31
Yes	25	28		42.6	
No	65	43		59.2	
α2 integrin			0.036		0.056
Weak expression	65	40		60.1	
Strong expression	25	31		45.6	
α3 integrin			0.019		0.033
Weak expression	62	36		63.4	
Strong expression	28	35		42.5	
α5 integrin			0.91		0.92
Weak expression	41	33		54.4	
Strong expression	49	38		55.1	
α6 integrin			0.017		0.0089
Weak expression	40	45		43.2	
Strong expression	50	26		66.6	
β1 integrin			0.21		0.20
Weak expression	47	30		58.0	
Strong expression	43	41		52.1	
β4 integrin			0.0027		0.0058
Weak expression	26	37		38.8	
Strong expression	64	34		66.2	

TABLE 2 Univariate and multivariate analysis of factors associated with recurrence-free survival after TUR in 161 patients with NMIBC

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Age (<60 years vs ≥60 years)	1.02 (0.62–1.56)	0.93	0.99 (0.51–1.92)	0.97
Gender (M vs F)	1.41 (0.77–2.58)	0.27	0.71 (0.35–1.43)	0.34
Preoperative urinary cytology (negative vs positive)	1.06 (0.58–1.51)	0.79	0.87 (0.49–1.55)	0.63
Multiplicity of tumour (single vs multiple)	1.33 (0.47–1.21)	0.24	0.64 (0.38–1.09)	0.10
Maximal tumour size (≤3 cm vs >3 cm)	2.13 (1.33–3.45)	0.001	1.80 (1.09–2.98)	0.023
Tumour type (papillary vs non-papillary)	1.71 (0.95–3.08)	0.071	0.76 (0.31–1.84)	0.54
T stage (Ta vs T1)	1.72 (1.08–2.78)	0.024	1.80 (1.09–3.01)	0.026
Grade (G1 or G2 vs G3)	1.35 (0.39–1.42)	0.37	0.81 (0.33–1.96)	0.64
CIS (negative vs positive)	2.22 (1.37–3.57)	0.035	0.76 (0.39–1.50)	0.43
Adjuvant intravesical instillation therapy (yes vs no)	1.55 (0.32–1.36)	0.24	0.85 (0.39–1.51)	0.49
α2 integrin (weak expression vs strong expression)	1.56 (0.98–2.50)	0.058	0.70 (0.41–1.19)	0.19
α3 integrin (weak expression vs strong expression)	1.64 (1.03–2.63)	0.035	0.66 (0.39–1.12)	0.13
α5 integrin (weak expression vs strong expression)	0.97 (0.61–1.56)	0.92	0.82 (0.45–1.51)	0.53
α6 integrin (weak expression vs strong expression)	1.89 (1.16–3.06)	0.010	0.60 (0.33–1.08)	0.084
β1 integrin (weak expression vs strong expression)	0.73 (0.46–1.18)	0.20	0.71 (0.41–1.23)	0.22
β4 integrin (weak expression vs strong expression)	1.91 (1.20–3.05)	0.0067	1.85 (1.03–3.31)	0.040

FIG. 1. Intravesical recurrence-free survival of 161 patients with primary NMIBC who underwent TUR.

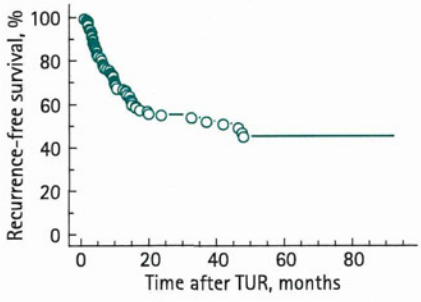
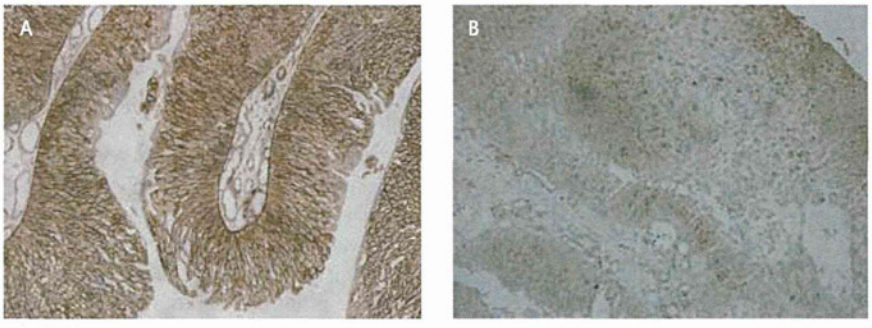


*FIG. 2. Typical outcomes of immunohistochemical staining of primary NMIBC with an antibody against β4 integrin subunit. **A**, NMIBC with weak expression of β4 integrin. **B**, NMIBC with strong expression of β4 integrin.*

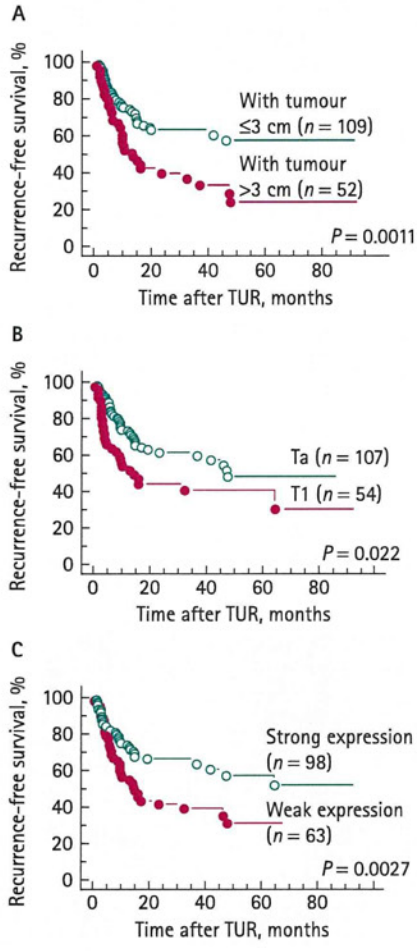


recurrence was detected in 71 of 161 patients (44.1%), and 1-, 3- and 5-year recurrence-free survival rates were 67.7%, 53.9% and 45.2%, respectively (Fig. 1). Table 1 presents the characteristics of 161 patients included in this study according to the presence or absence of intravesical recurrence. Of several variables examined in this study, maximal tumour size and concomitant CIS were significantly related to the incidence of intravesical recurrence. Furthermore, there was a significant association between the expression level of α2, α3, α6 or β4 integrin subunits in NMIBC and the incidence of intravesical recurrence after TUR, despite the lack of significant impact of the expression levels of α5 and β1 subunits on the incidence of intravesical recurrence.

We then evaluated the significance of several factors, including the expression patterns of integrin subunits, in the probability of intravesical recurrence. As shown in Table 2, univariate analysis using the Cox proportional hazards regression model showed that expression levels of the α3, α6 and β4 subunits were significant variables associated with intravesical recurrence-free survival, while maximal tumour size, pathological T stage and concomitant CIS were also significant. In addition, expression level of β4 integrin subunit, maximal tumour size and pathological T stage appeared to be independent predictive factors of intravesical recurrence on multivariate analysis (Table 2). Representative findings of immunohistochemical study for detecting β4 integrin expression are shown in Fig. 2, and recurrence-free survival curves according to maximal tumour size, pathological T stage and expression level of β4 integrin, are presented in Fig. 3. There were significant differences in recurrence-free survival with respect to all three of these factors.

To characterize more precisely the postoperative clinical features of NMIBC, we categorized patients who were positive for the three independent risk factors for intravesical recurrence (maximal tumour size, pathological T stage and β4 integrin expression level). During the observation period of this series, intravesical recurrence developed in 10 of 49 patients who were negative for all risk factors (20.4%), 31 of 68 positive for a single risk factor (45.6%), and 30

FIG. 3. Intravesical recurrence-free survival of patients with primary NMIBC who underwent TUR with regard to maximal tumour size (A), pathological T stage (B) and expression level of β4 integrin subunit (C).



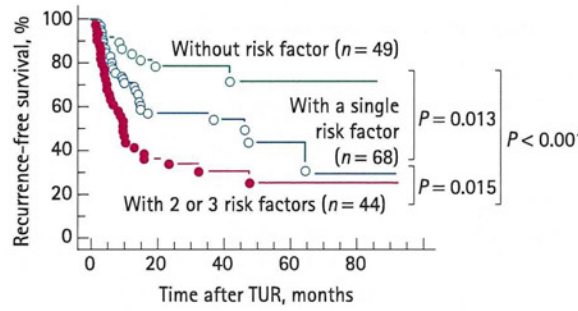


FIG. 4. Intravesical recurrence-free survival of patients with primary NMIBC with regard to the following independent risk factors for intravesical recurrence after TUR: maximal tumor size; pathological T stage; and expression level of β4 integrin subunit.

of 44 positive for two or three risk factors (68.2%). As shown in Fig. 4, there were significant differences in intravesical recurrence-free survival among these three subgroups.

DISCUSSION

To date, a number of investigators [3–7] have evaluated variables that precisely predict the probability of intravesical recurrence after TUR of NMIBC, however, the outcomes of these studies failed to provide consistent findings. Furthermore, considering the diverse genetic, as well as biological, characteristics of NMIBC [29], it may not be sufficient to assess clinicopathological factors alone for stratifying patients with NMIBC into risk groups with respect to intravesical recurrence after TUR. In the present study, therefore, we evaluated the values of integrins, a family of transmembrane heterodimers, which have been shown to be involved in a wide variety of pathophysiological cell functions [19–22], in addition to conventional clinicopathological variables for predicting prognosis in 161 consecutive patients undergoing TUR for NMIBC.

In the present series, 44.1% of the patients experienced intravesical disease recurrence, achieving the 5-year recurrence-free survival rate of 45.2%, which is comparable to those in previous studies [1–3]. Differences in characteristics, including the expression patterns of integrin subunits, between patients with and without intravesical recurrence, were then analysed. Among several conventional factors examined in this series, maximal tumour size and concomitant CIS were significantly associated with the incidence of intravesical recurrence. Several previous studies found results similar to those in the present study [3–7]. For example, Millan-Rodriguez *et al.* [7] reported a greater incidence of intravesical recurrence in patients with NMIBC who were diagnosed as having multiple tumours, tumours >3 cm and/or concomitant CIS. In addition, of the integrin subunits investigated in this series, the strong expression of α2 and α3 as well as the weak expression of α6 and β4 subunits were shown to have a significant impact on the incidence of intravesical recurrence. There have been only a few studies analysing the expression patterns of these subunits in NMIBC specimens [25,26,28] and the prognostic significance of integrin expression in NMIBC is still not known. It would be necessary to conduct a prospective assessment of a larger number of patients in order to draw a definitive conclusion on this issue.

To develop a reliable prediction system for intravesical recurrence after TUR of NMIBC, which is potentially useful for determining post-TUR follow-up as well as for treatment schedules, the effects of conventional prognostic variables and expression levels of integrin subunits on time to intravesical recurrence after TUR were investigated. Univariate analysis identified expression levels of α3, α6 and β4 subunits, in addition to tumour size, pathological T stage and concomitant CIS, as significant predictors of intravesical recurrence. Using multivariate analysis, however, only β4 integrin expression, tumour size and pathological T stage appeared to be independently associated with intravesical recurrence-free survival. As indicated above, tumour size and T stage are useful prognostic indicators in patients with NMIBC undergoing TUR [3–7]. To our knowledge, however, the present study is the first to demonstrate the independent significance of β4 integrin expression as a predictor of intravesical recurrence of NMIBC, although some studies [23,26] have suggested the important role of the β4 integrin subunit in disease progression of muscle-invasive bladder cancer. Furthermore, Clarke *et al.* [30] showed that forced expression of β4 integrin in a colon cancer cell line resulted in the induction of p21, which was shown to have a negative effect on intravesical recurrence of NMIBC in our previous study [18]. Collectively, these findings indicate that the linkage of β4 integrin to a signalling pathway involved in cell-cycle regulation through the induction of p21 expression may be involved in the molecular mechanism underlying the intravesical recurrence of NMIBC.

To allow more precise risk stratification for postoperative intravesical recurrence in an individual patient with NMIBC, combined assessment of the three independent predictors for intravesical recurrence

identified by multivariate analysis was performed. When the patients were classified into three subgroups, negative for all risk factors, positive for a single risk factor and positive for two or three risk factors, there were significant differences in recurrence-free survival among these three groups. These findings suggest that it would be useful to develop a novel system that can more accurately predict intravesical recurrence of NMIBC after TUR based on the consideration of the three risk factors (maximal tumour size, pathological T stage and β4 integrin expression). Moreover, the usefulness of the combined use of these three factors could contribute to further refinement of the system. However, a prospective study including additional information with more potential molecular markers involved in the intravesical recurrence of NMIBC will be required before a definitive conclusion can be drawn.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **NMIBC**, non-muscle-invasive bladder cancer; **TUR**, transurethral resection; **CIS**, carcinoma *in situ*.

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Original Article: Clinical Investigation

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

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Objectives: We carried out a prospective, randomized, controlled trial to investigate the efficacy and safety of both induction and maintenance therapy with intravesical instillation of bacillus Calmette-Guérin (BCG) for high-risk non-muscle invasive bladder cancer (NMIBC).

Methods: Intravesical instillation of 80 mg Tokyo strain was given to patients with high-risk NMIBC, including carcinoma *in situ* (CIS), once weekly for eight consecutive weeks as induction therapy. Patients who achieved complete response (CR) were randomly assigned to either the maintenance group or the observation group.

Results: A total of 90 patients were enrolled. After induction therapy, 75% of the patients achieved CR and 53 of them were enrolled in the randomized comparative phase. A total of four maintenance instillations were given. Median follow-up was 26.5 and 28.7 months after randomization in the maintenance and the observation group, respectively. Although it was not statistically significant, the 2-year recurrence-free survival rate in the maintenance group (95.8%) was higher than that in the observation group (74.1%, $P = 0.078$). Univariate analysis identified maintenance therapy as a significant factor influencing recurrence. During induction therapy, 82.2% of patients experienced urination-related adverse drug reactions, but most events were not serious. There were fewer adverse drug reactions with maintenance therapy than with induction therapy. Neither induction therapy nor maintenance therapy reduced patients' quality of life (QOL).

Conclusions: These findings show high levels of efficacy and safety of BCG induction treatment for high-risk NMIBC, and suggest that the number of maintenance instillations could probably be reduced without reducing treatment efficacy or influencing QOL.

Key words: Bacillus Calmette-Guérin, carcinoma *in situ*, maintenance instillation, non-muscle invasive bladder cancer, randomized trial.

Introduction

Intravesical instillation of bacillus Calmette-Guérin (BCG) has been reported to have an advantage over intravesical chemotherapy in terms of therapeutic benefit and prophylaxis of non-muscle invasive bladder cancer (NMIBC).[1,2] A meta-analysis study also showed that this treatment method reduced the risk of progression of NMIBC.[3] Intravesical instillation of BCG is currently regarded as a standard modality for the treatment of high-risk Ta, T1 bladder cancer and carcinoma *in situ* (CIS) of the bladder, as well as for the prophylaxis of recurrence after the resection of high-risk Ta, T1 bladder cancer.[4,5] The South West Oncology Group (SWOG) randomized clinical trial showed that 5-year recurrence-free survival and 5-year progression-free survival were higher in patients who received BCG induction therapy followed by maintenance therapy than those who received only BCG induction therapy.[6] A meta-analysis showed that only BCG instillation as maintenance therapy

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prevented the progression of NMIBC.[7] Thus, BCG maintenance therapy is recommended to prevent recurrence or progression and to prolong survival in patients with high-risk NMIBC.[4,5] In the SWOG randomized study, the maintenance period was longer than two years and a total number of 21 maintenance instillations were given, although a 3-week maintenance schedule seems to be regarded as the standard maintenance instillation method. This might be one of the reasons why only a small number of patients completed the maintenance BCG instillation. It shows that efforts should be made to develop another BCG maintenance schedule.

In this time, we carried out a prospective, randomized, comparative study to investigate the efficacy and safety of both induction and maintenance BCG therapy, as well as another schedule of maintenance instillation for high-risk Ta, T1 cancer and CIS of the bladder.

Methods

This is a multicenter, prospective, open-label, randomized, comparative study. The present clinical trial protocol was approved by the institutional review board of each participating institution. Patients with histologically-confirmed Ta, T1 transitional cell carcinoma or CIS of the bladder were eligible to participate in the present study. In the case of Ta and T1, the tumor had to be diagnosed as not completely resectable by transurethral resection.

Other inclusion criteria were: (i) performance status (PS) of 0 to 2; (ii) age between 20 and 79 years; (iii) able to undergo assessment, such as cystoscopy and urinary cytology, on a regular basis; (iv) presence of at least one cystoscopically measurable or evaluable lesion in the case of unresectable NMIBC; and (v) intact function of the main organs (i.e. bone marrow, liver, kidney, heart and lung) Exclusion criteria were: (i) strongly positive tuberculin reaction or active tuberculous lesion; (ii) history of BCG intravesical instillation therapy; (iii) severe bladder irritation before the start of drug administration; (iv) intravesical instillation therapy with an anticancer drug within 3 weeks before the start of BCG administration; (v) history of bladder-sparing treatment for invasive bladder cancer; (vi) history of upper urinary tract carcinoma; and (vii) active double cancer or serious medical complications. Before treatment, patients provided a history and underwent physical examination, urinalysis, urine cytology examination, complete blood count, blood urea nitrogen, serum creatinine, liver function tests and electrocardiography. Chest X-ray and excretory urography were also carried out.

All eligible patients had to receive a full explanation of this study and sign an informed consent form before registration by facsimile to the central registration center. Induction therapy with 80 mg of BCG Tokyo strain started within 4 weeks after bladder biopsy or TUR for histological diagnosis. Patients were required to hold their urine for approximately 2 h after instillation. BCG treatment was repeated weekly for eight consecutive weeks. In the case of adverse drug reactions (ADR), administration could be delayed, but not for longer than 3 weeks from the scheduled administration day. Efficacy was evaluated at 4–12 weeks after the completion of this induction therapy. Patients who achieved CR on the induction therapy were required to receive a full explanation of the randomized study before re-registration at the same central registration center as for the induction therapy. The registered patients were randomly assigned to either the maintenance or the observation group. Maintenance therapy involved a single intravesical instillation of BCG Tokyo strain 80 mg within 3 months after the randomization, followed by instillations at 3, 6 and 9 months after the initial dose (a total of 4 doses). The dosing interval of maintenance therapy was set as 3 months based on the approved regimen of a similar drug that is already on the market in the USA. The total number of maintenance doses was determined as follows; the maximum number of doses was considered to be 12 which is 1.5 times the approved regimen of BCG Tokyo strain in Japan and because eight doses would be given as induction therapy, an additional four doses were to be given as maintenance therapy.

Patients in both groups underwent urinalysis, urinary cytology and cystoscopy 2 months after randomization. These examinations were repeated for observing the presence or absence of recurrence every 3 months for the first

Table 1 Patient characteristics

	No. patients
Enrolled patients	90
Evaluable patients	84
Sex	
Male	68
Female	16
Age	
Younger than 70 years	39
70 years or older	45
Performance status	
0	80
1, 2	4
Tumor status	
Primary tumor	60
Recurrent tumor	24
Stage	
CIS	74
Ta,T1 tumor	10
Smoking habit	
No	28
Yes	56

CIS, carcinoma *in situ*.

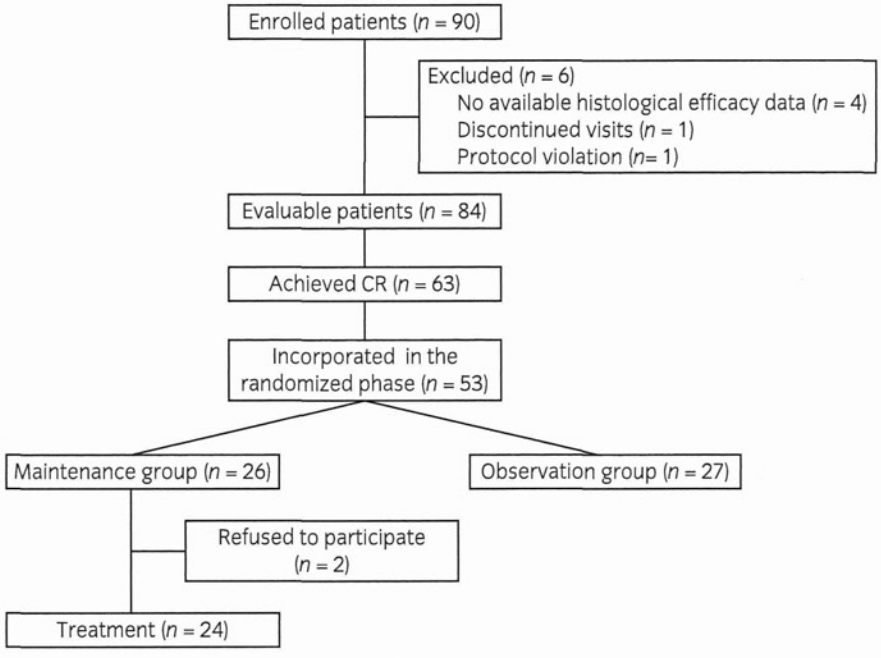

Fig. 1 CONSORT flow diagram. CR, complete response.

3 years, and thereafter every 6 months. Clinical chemistry examination was carried out before the induction therapy, and before the fifth instillation and 2 weeks after the eighth instillation of induction therapy. In the maintenance group, clinical laboratory tests were carried out at 2, 5, 8 and 11 months after randomization. ADR were monitored at every visit and assessed according to the Common Terminology Criteria of Adverse Events version 3.0 (CTC AE v3.0). ADR that were difficult to grade by CTC AE, such as pain on urination and difficulty to urinate, were classified into grade 1 (mild), grade 2 (moderate) and grade 3 (severe). Whenever disease progression was suspected, imaging tests such as CT were carried out. Quality of life (QOL) was assessed according to the Japanese version of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Core 30 (EORTC QLQ-C30) v2.0[8] before the induction therapy, after the fifth instillation of induction therapy, 4 weeks after the completion of induction therapy and 14 months after randomization. Before using the QOL scale, authorization was acquired by the prescribed procedure.

The primary endpoint was the efficacy of induction therapy, and the secondary endpoints were recurrence-free survival, progression-free survival, overall survival, QOL and the frequency and severity of ADR in both groups. The efficacy was assessed according to the third edition of the General Rules for Clinical and Pathological Studies on Bladder Cancer.[9] For Ta and T1 cancer, CR was defined as the complete clinical disappearance of target legions with negative biopsy and urine cytology. For CIS, CR was defined as negative biopsy and urine cytology. Recurrence was examined with cystoscopy and urine cytology, and then confirmed histologically. Disease progression was defined as the emergence of muscle invasive cancer or distant metastasis. The last observation day was 31 March 2007, when the present study was closed.

The sample size was determined as follows: CIS is the rarest type, representing just 4% of bladder cancer. Because there have been few reports of maintenance therapy given after BCG treatment for bladder cancer, as in the present study, it was considered difficult to determine the sample size from a statistical viewpoint. Therefore, the sample size was set as 30 patients in each group (a total of 60 patients) in the comparative phase of the study, taking feasibility into consideration. In a previous clinical study using BCG Tokyo strain, the CR rate in partly unresectable Ta, T1 bladder cancer and CIS of the bladder was 70.1%.[10] If the number of patients required for random assignment is 60, then 82.3 patients must be enrolled in induction therapy, taking an estimation error of 10% into account. Taking patients who withdrew from the study and those who refused to participate in the comparative phase of study into consideration, the number of patients enrolled was set as 90.

The significance of differences in patient background factors between the two groups was examined by the χ^2-square test. Recurrence-free, progression-free and overall survival curves were calculated by the Kaplan–Meier method and statistical significance was determined by the log–rank test. The Cox proportional hazard model was used to carry out univariate and multivariate analysis. QOL was evaluated using EORTC QOL-C30 scoring manual and examined by the paired *t*-test. Differences were considered to be significant at $P < 0.05$.

Table 2 Patient characteristics of the maintenance and observation groups

	Maintenance group	Observation group	*P*-value
Sex			
Male	19	21	0.8253
Female	5	6	
Age			
Younger than 70 years	9	14	0.3039
70 years or older	15	13	
Performance status			
0	23	25	0.9162
1, 2	1	2	
Tumor status			
Primary tumor	17	20	0.9558
Recurrent tumor	7	7	
Stage			
CIS	21	25	0.8897
Ta,T1 tumor	3	2	
Smoking habit			
No	9	7	0.5573
Yes	15	20	
Median follow-up period (range)	26.5 (13.9–40.7)	28.7 (14.9–51.7)	0.7199

CIS, carcinoma *in situ*.

Results

A total of 90 patients were enrolled between October 2002 and June 2005. The characteristics of enrolled patients are shown in Table 1. As shown in Figure 1, efficacy was evaluated for 84 patients. For the induction therapy, 6 of 10 patients with Ta and T1 cancer and 57 of 74 patients with CIS of the bladder achieved CR, resulting in CR rates of 60.0% and 77.0%, respectively. The overall CR rate was 75.0%. Among 63 patients who achieved CR, 53 patients were enrolled in the maintenance therapy phase and randomized: 26 to the maintenance group and 27 to the observation group. Two patients randomized to the maintenance group withdrew their consent before the start of maintenance instillation (Fig. 1). There were no significant differences in patient characteristics between the two groups (Table 2). The number of doses given to 24 patients randomized to the maintenance group was one in two patients (8.3%), two in three patients (12.5%), three in one patient (4.2%) and four in 18 patients (75.0%), showing a good completion rate of the maintenance regimen. There was not a significant difference in the follow-up period between the two groups. Recurrence was confirmed in one patient in the maintenance group and seven patients in the observation group, which showed 2-year recurrence-free survival rates of 95.8% and 74.1%, respectively. This indicates a lower recurrence rate in the maintenance therapy than in the observation group (Fig. 2), but the difference between the groups was not significant ($P = 0.078$). Disease progression was not seen in the maintenance group and was seen in one patient in the observation group. There was no significant difference in progression free survival between the groups ($P = 0.383$). Two patients each in the maintenance group and the observation group died during the study. One of the two

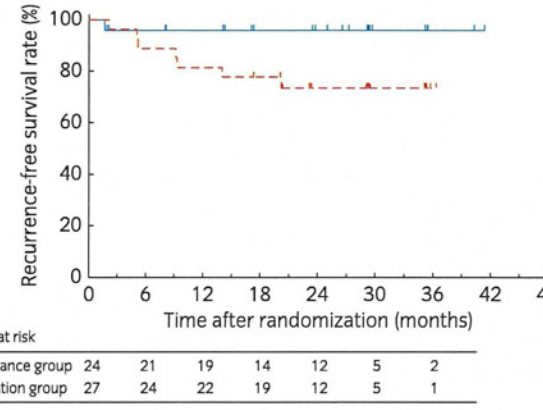

Fig. 2 Recurrence-free survival curves after randomization into two groups. A lower recurrence rate was observed in the maintenance group than in the observation group, but the difference between the two groups was not significant ($P = 0.078$). ——, Maintenance group ($n = 24$); - - - -, observation group ($n = 27$).

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

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Appendix

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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?".

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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

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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

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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

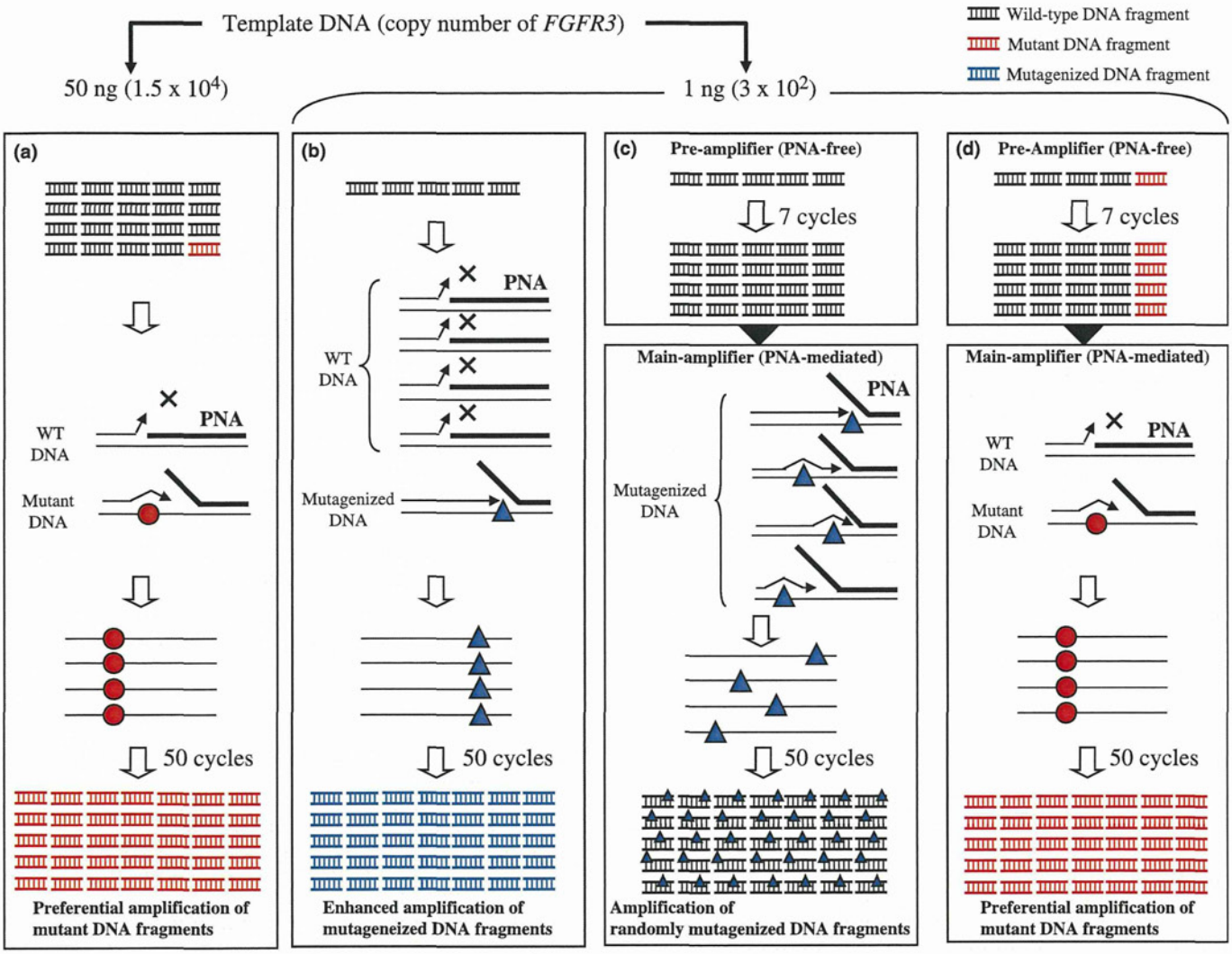

Fig. 1. Schematic diagram of the one-step and two-step peptide nucleic acid (PNA)-mediated PCR clamping. The feasibility of PNA-mediated PCR clamping was highly influenced by the amount of the template DNA. (a) Fifty nanograms of genomic DNA containing a low proportion of mutated DNA was used as a template. 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.

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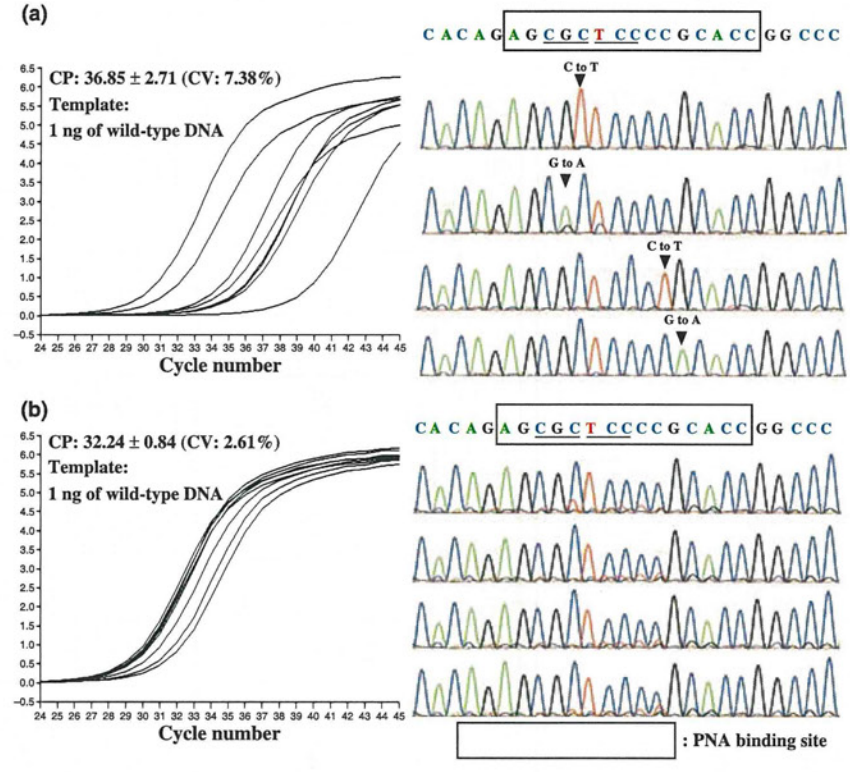

Fig. 2. Two-step peptide nucleic acid (PNA)-mediated real-time PCR clamping decreased the variance of crossing points (CP) and avoided the enhanced misincorporation of dNTPs. In the experiment for fibroblast growth factor receptor (*FGFR*)-3 exon 7, 1 ng of genomic DNA containing only wild-type *FGFR3* 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%