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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 *FGFR*3 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 180g 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 *FGFR*3 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 *FGFR*3 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 *FGFR*3 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

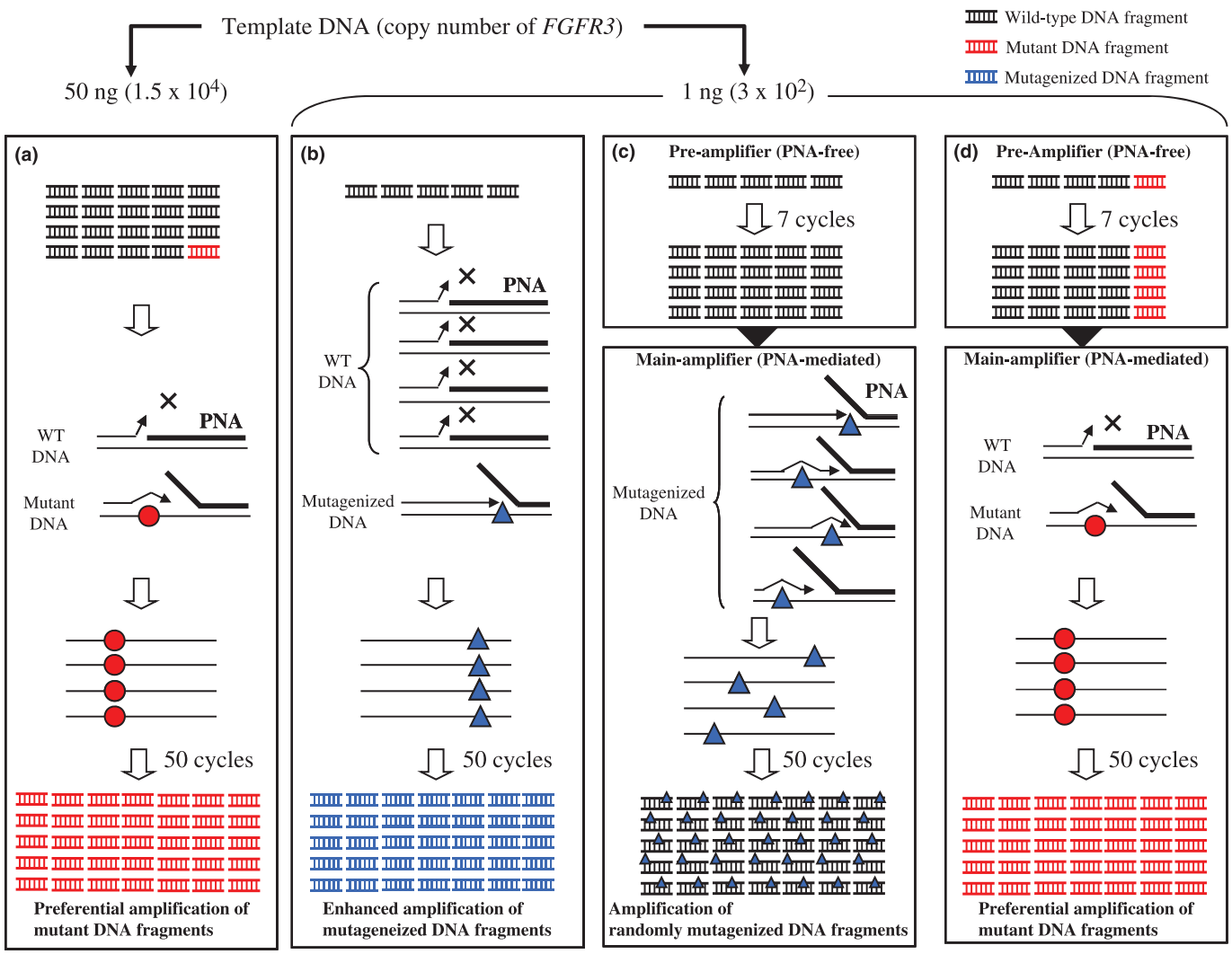

Fig. 1. Schematic diagram of the one-step and two-step peptide nucleic acid (PNA)-mediated PCR clamping. The feasibility of PNA-mediated PCR clamping was highly influenced by the amount of the template DNA. (a) Fifty nanograms of genomic DNA containing a low proportion of mutated DNA was used as a template.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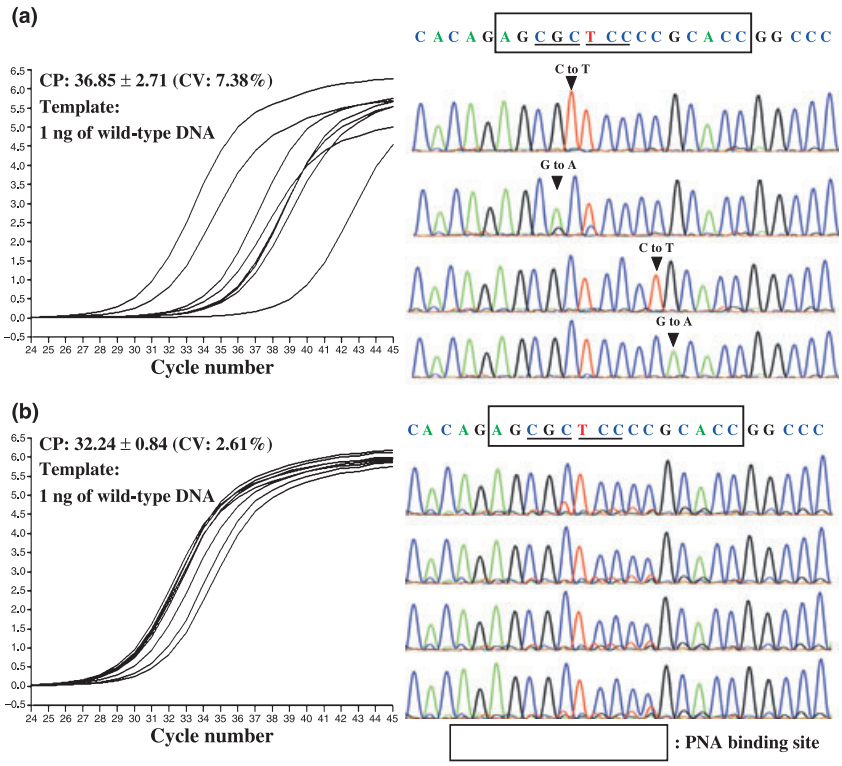

Fig. 2. Two-step peptide nucleic acid (PNA)-mediated real-time PCR clamping decreased the variance of crossing points (CP) and avoided the enhanced misincorporation of dNTPs. In the experiment for fibroblast growth factor receptor (*FGFR*)-3 exon 7, 1 ng of genomic DNA containing only wild-type *FGFR3* 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 *FGFR*3 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 *FGFR*3 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 *FGFR*3 mutations in 45 NMIBC samples, 24 (53.3%) tumors harbored activating mutations of *FGFR*3, and their correlations with the clinicopathological variables are summarized in Table 2. No variables showed significant correlation with *FGFR*3 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 *FGFR*3 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 *FGFR*3 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 *FGFR*3 mutation in the urine samples was 62%

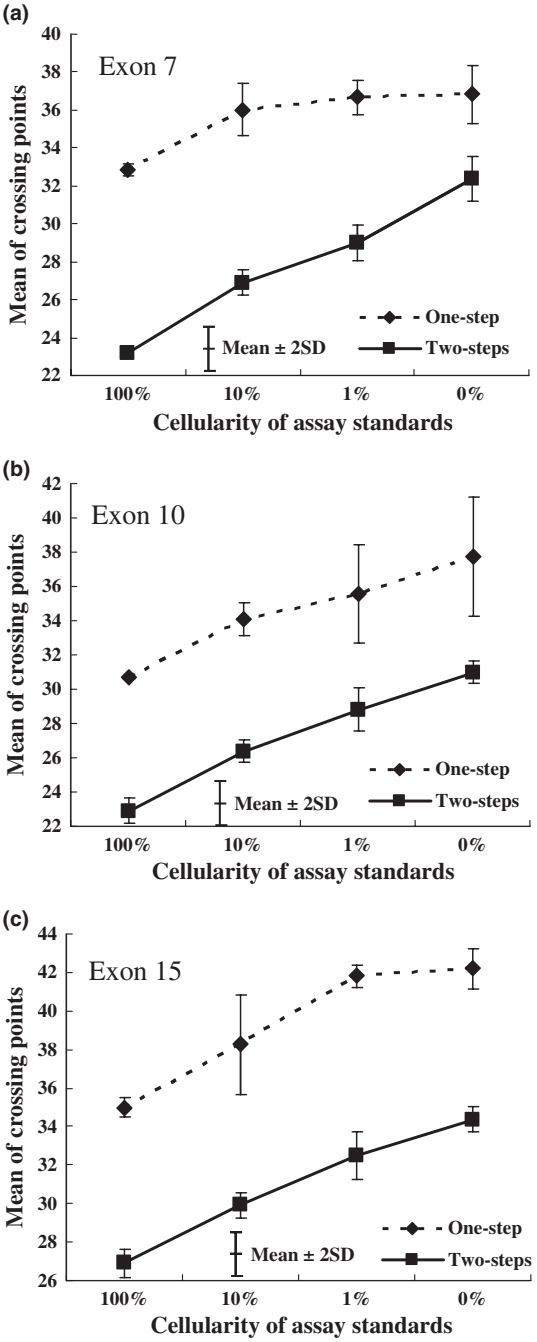

Fig. 3. Validation for sensitive detection of fibroblast growth factor receptor (*FGFR*)-3 mutation in trace amounts of the template DNA. (a) One nanogram of genomic DNA of four assay standards with 0, 1, 10, and 100% of tumor cellularity was amplified in octaplicate samples (n = 8) under the conditions 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 *FGFR*3 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 *FGFR*3 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).

*FGFR*3 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

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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 *FGFR*3 mutations in the primary tumor, the levels of *FGFR*3 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 *FGFR*3 mutations in the voided urine samples during the follow-up period after the initial TURBT. *FGFR*3 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 *FGFR*3 mutations in the recurrent and non-recurrent cases were 88.9% (8/9) and 41.7% (5/12), respectively. *FGFR*3 mutations were not detected 1 month after the initial TURBT in all cases. *FGFR*3 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 *FGFR*3 mutations except for five urine samples obtained before the initial TURBT. The frequencies of *FGFR*3 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 *FGFR*3 mutations in the postoperative urine samples were defined as the positive or negative rates of *FGFR*3 mutations in recurrent or non-recurrent cases, respectively. In cases with *FGFR*3 mutation in the primary tumors, the sensitivity and specificity of urine *FGFR*3 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 *FGFR*3 mutations in the tumors. In analysis of cases with wild-type *FGFR*3 in the primary tumors, *FGFR*3 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 *FGFR*3 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).

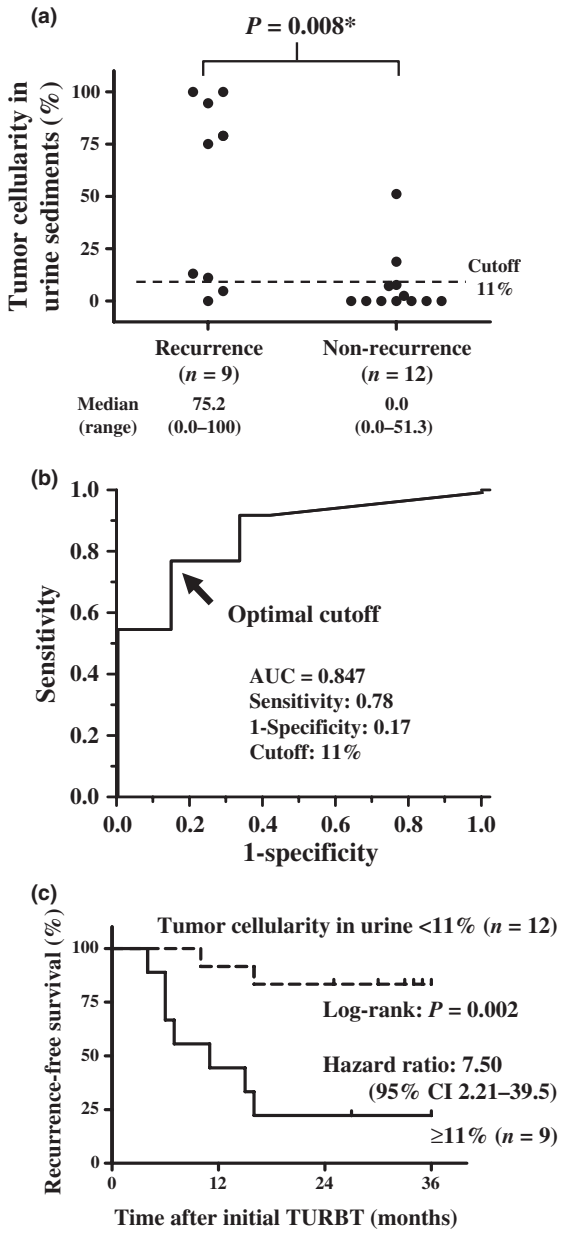

Fig. 4. The tumor cellularity in the preoperative urine predicted the risk of intravesical recurrence in patients with fibroblast growth factor receptor (*FGFR*)-3-mutated bladder urothelial carcinoma (UC). (a) The tumor cellularities in the preoperative urine sediments were plotted in the recurrent (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.

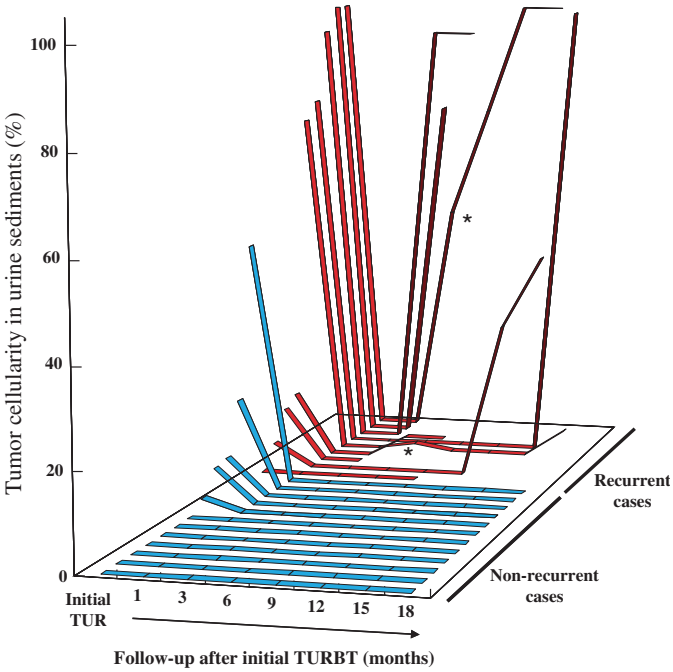

Fig. 5. The time-course analysis of tumor cellularity in the voided urine sediments in cases with mutated-fibroblast growth factor receptor (*FGFR*)-3. Tumor cellularities serially determined after initial transurethral resection of bladder tumor (TURBT) are plotted on 3-D line chart. All cases had *FGFR3* 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 *FGFR3* 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

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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%.

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Disclosure Statement

None.

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