quency of this mutation in superficial bladder cancers. Recently, the peptide nucleic acid (PNA)-mediated PCR clamping technique has been developed to improve assay sensitivity for detecting mutations in samples including various tissues, body fluids and stool [4,5]. PNA is a synthetic DNA analog in which the phosphodiester backbone is replaced by a peptide-like repeat of the (2-aminoethyl)-glycine chain [6]. A perfectly matched PNA/DNA hybrid has higher thermal stability than the corresponding DNA/DNA hybrid, hence single base-pair mismatch results in a decrease of Tm at 9–16 °C. On PCR, PNA hybridized with the target sequence can inhibit primer annealing or chain elongation without interfering with reactions of mismatched template DNA; therefore, so-called PNA-mediated PCR clamping induces preferential amplification of mutant DNA fragments even in the presence of an excess amount of wild-type DNA. In the present study, we devised a PNA-mediated real-time PCR clamping technique and established a rapid, simple and sensitive method for detecting *FGFR3* mutations in mutational hotspots in exons 7, 10 and 15 in bladder tumors and voiding urine sediments.

Materials and methods

Samples and DNA extraction. Tumor tissues and preoperative voiding urine samples were obtained from 19 patients with bladder UCC who underwent TUR. Tumors were histologically staged according to the 1997 UICC TNM classification system [7] and graded according to the 1973 WHO classification system [8]. Voiding urine samples from 20 patients with chronic cystitis were assayed as negative controls. Genomic DNA was extracted by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation as described previously [9]. Genomic DNA concentrations were determined by ultraviolet measurement with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Cell lines. Genomic DNAs extracted from cell lines, UM-UC-14 [10], MGHU3 and J82 were used as assay standards. These cell lines harbored representative *FGFR3* mutations such as homozygous mutation at codon 249 (TCC->TGC) in exon 7 (UM-UC-14), homozygous mutation at codon 375 (TAT->TGT) in exon 10 (MGHU3), and heterozygous mutation at codon 652 (AAG->GAG) in exon 15 (J82), respectively [11,12]. MGHU3 and J82 were generous gifts from Dr. H. LaRue (Laval University Cancer Research Centre, Quebec, Canada) and Dr. Y. Nishiyama (Kyoto University, Kyoto, Japan), respectively.

Primers and PNA for real-time PCR. Primers and PNAs were synthesized by FASMAC. CO., Ltd. (Kanagawa, Japan) and their sequences are listed in Table 1. In exon 7 of *FGFR3*, two types of different point mutations were reported in bladder UCC such as C to T transition at codon 248 substituting cystein for arginine (R248C) and C to G transversion at codon 249 substituting cystein for serine (S249C). PNA was designed as a 15-mer probe which completely matched the bottom strand of the wild-type sequence spanning from the second position of codon 247 through the first position of codon 252 (Fig. 1). Primers and PNAs for exons 10 and 15 were designed according to the same principle as that for exon 7.

PNA-mediated real-time PCR clamping. PNA-mediated real-time PCR clamping was performed with LightCycler (Roche Diagnostics, Mannheim, Germany) in 20 μl reaction volume containing genomic DNA ranging from 50 to 1 ng, 10 μl of QuantiTect PCR master mix (Qiagen, Valencia, CA) containing *Taq* DNA polymerase and SYBR® green I fluorescent dye, 500 nmol/L of each primer and various concentrations of

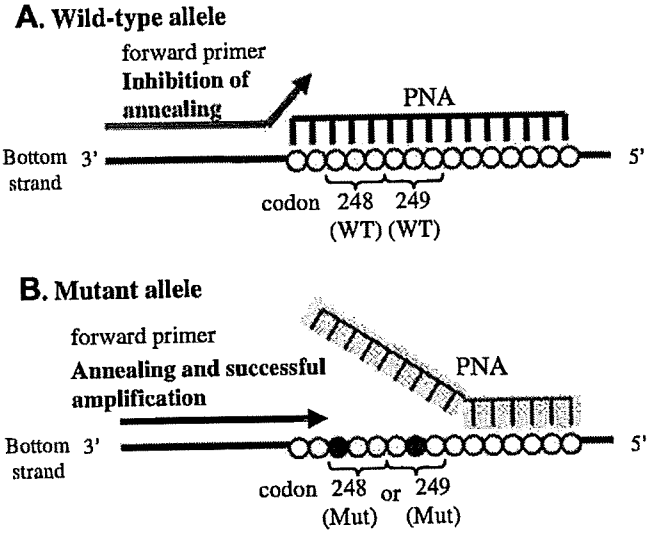

Fig. 1. The schematic concept of PNA-mediated PCR in *FGFR3* exon 7. The 15-mer PNA was designed to bind to the bottom strand of wild-type sequence spanning mutational hotspots at codon 248 or 249 in exon 7 of the FGFR3 gene. Forward PCR primer was designed to partially overlap the PNA binding site. A PNA/DNA hybrid with a perfect match suppressed annealing of the forward primer and subsequent amplification of wild-type alleles (A), while a single base pair mismatch of the PNA/DNA hybrid led to decreased thermal stability (ΔT_m 9–16 °C), resulting in preferential amplification of mutant alleles (B).

Table 1
Sequences of primers and PNAs used in this study

FGFR3	Name	Sequences	Product length (bps)	PNA concentration (μM)	PNA binding step (°C)	Annealing step (°C)
Exon 7	7F	5′-TGA GCG TCA TCT GCC CCC ACA GAG-3′	182	0.4	72	64
	7R	5′-GGG CCC ACC TTG CTG CCA TTC A-3′				
	PNA-7	H2N-AGC GCT CCC CGC ACC-N2H				
Exon 10	10F	5′-CCA GGC CTC AAC GCC CAT GTC TTT-3′	95	1	67	58
	10R	5′-ACC CCG TAG CTG AGG ATG CCT GCA-3′				
	PNA-10	H2N-CAT ACA CAC TGC CCG C-N2H				
Exon 15	15F	5′-GCA ATG TGC TGG TGA CCG AG-3′				
	15R	5′-CGG GCT CAC GTT GGT CGT CT-3′	108	2	70	60
	PNA-15	H2N-GGT CGT CTT CTT GTA GT-N2H				

Abbreviation: PNA, peptide nucleic acid.

PNAs. Conditions of real-time PCR were as follows; a first denaturing step of 95 °C for 15 min, amplification step of 45 cycles consisting of heat denaturation at 94 °C for 15 s, PNA/wild-type DNA binding step for 5 s, primer annealing step for 20 s and extension step at 72 °C for 20 s followed by a final cooling step of 40 °C for 30 s. The optimized PNA concentrations and reaction temperatures of PNA/WT binding and annealing steps varied for each exon as listed in Table 1. Amplification curves were obtained and the crossing point (CP) was calculated according to fit point method on LCDA (LightCycler Data Analysis)™ software version 3.5. In analysis of clinical samples, genomic DNAs extracted from cell lines were serially diluted with normal genomic DNA at various proportions (100%, 10%, 1%, and 0%) and used as assay standards.

Direct sequencing analysis. Amplified DNA fragments after PNA-mediated real-time PCR clamping were treated by a mixture of exonuclease I and shrimp alkaline phosphatase using ExoSAP-IT (USB Corporation, Cleveland, Ohio) and subjected to a cycle sequencing reaction with a BigDye v3.1 terminator sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Sequencing products were purified using a Sephadex G-50 Superfine (Amersham, Uppsala, Sweden) column centrifuge with MultiScreen HV plates (Millipore, Bedford, MA) and subjected to ABI PRISM 3100 Genetic Analyzer.

Results

Validation of assay sensitivity by PNA-mediated real-time PCR clamping using serially diluted assay standards

We evaluated assay sensitivity using DNAs extracted from cell lines harboring *FGFR3* mutations. In the analysis of exon 7, the proportion of UM-UC-14-derived DNA to normal genomic DNA, e.g., tumor cellularity, was adjusted to 100%, 10%, 1%, and 0%. Fifty nanograms of genomic DNA were used as templates for PNA-mediated real-time PCR clamping. The yield of the PCR product was indicated as the intensity of fluorescence emission from SYBR Green I dye bound to double-strand DNA fragments which increases as the cycle of PCR proceeds (Fig. 2A). The number of cycles reaching a certain level of fluorescence intensity (threshold line) was indicated as the CP. In PCR using templates with different tumor cellularities, CPs were in reverse correlation with tumor cellularities. Direct sequencing of the amplified DNA fragments revealed that the products of 100%, 10%, and 1% standard templates showed a mutation of C to G transversion at codon 249 (TCC->TGC) in exon 7 of *FGFR3* (Fig. 2B). No amplification product was obtained in PCR using distilled water as a negative control, while PCR using wild-type DNA (tumor cellularity: 0%) as the template showed delayed CP, but the amplification profile was similar to mutation-positive samples (Fig. 2A). In direct sequencing of the amplified DNA fragments, no mutation was found at codon 249 while the background signal within the PNA-binding region was increasing (Fig. 2B). This phenomenon implied that PNA-mediated PCR clamping facilitated the misincorporation of nucleotides to the PNA binding site in the absence of *FGFR3* mutations in the template.

Assay sensitivity of PNA-mediated real-time PCR clamping is defined by the amount of template DNA, the decrease of which induced misincorporation of dNTPs to PNA binding sites

Further experiments were carried out to validate assay conditions to reduce the rate of misincorporation and improve assay specificity. Assay standards with tumor cellularities ranging from 100%, 10%, 1%, and 0% were amplified in octaplicate samples ($n = 8$) by PNA-mediated real-time PCR clamping. Amounts of template DNA were

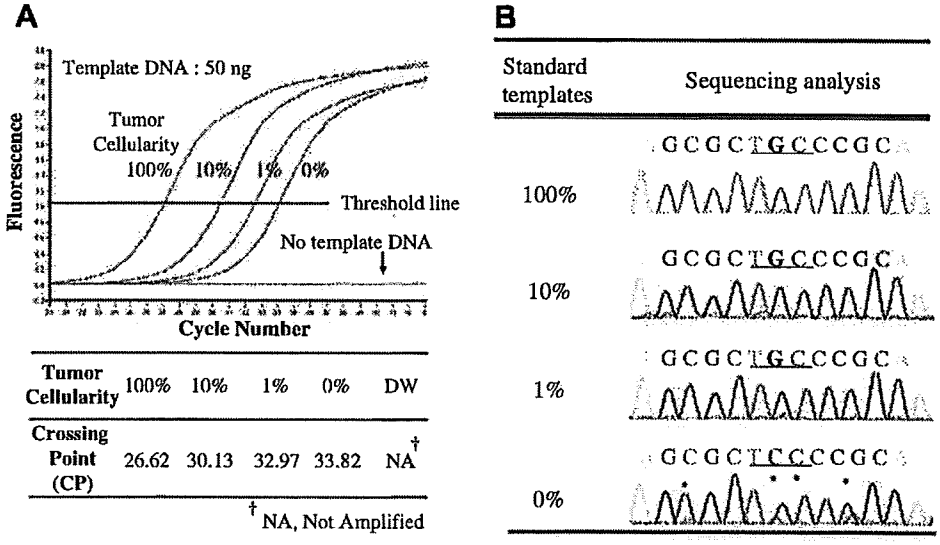

Fig. 2. Amplification of serially diluted standard templates by PNA-mediated real-time PCR clamping in *FGFR3* exon 7. Genomic DNA harboring a homozygous S249C mutation was serially diluted with wild-type DNA. Concentrations of mutated DNA in the sample were adjusted as follows: 100%, 10%, 1%, and 0%. (A) Fifty nanograms of template DNA were amplified using PNA-mediated real-time PCR clamping. The crossing points obtained by the threshold line at the fluorescence level of 1.0 were 26.62, 30.13, 32.97 and 33.83, respectively. (B) Subsequent DNA sequencing analysis revealed that PCR fragments of 100%, 10%, and 1% standard showed TCC to TGC mutations at codon 249, whereas that of 0% standard showed wild-type sequence, while background signals increased in the sequencing profile. Underlined letters indicate codon 249. Asterisks indicate the increase of background signals.

changed in each run from 50 ng, 10 ng to 1 ng. Amplification profiles and CPs of the assay were compared between runs (Fig. 3A and B). In the condition using 50 ng of genomic DNA as the template, CVs of the CPs were relatively

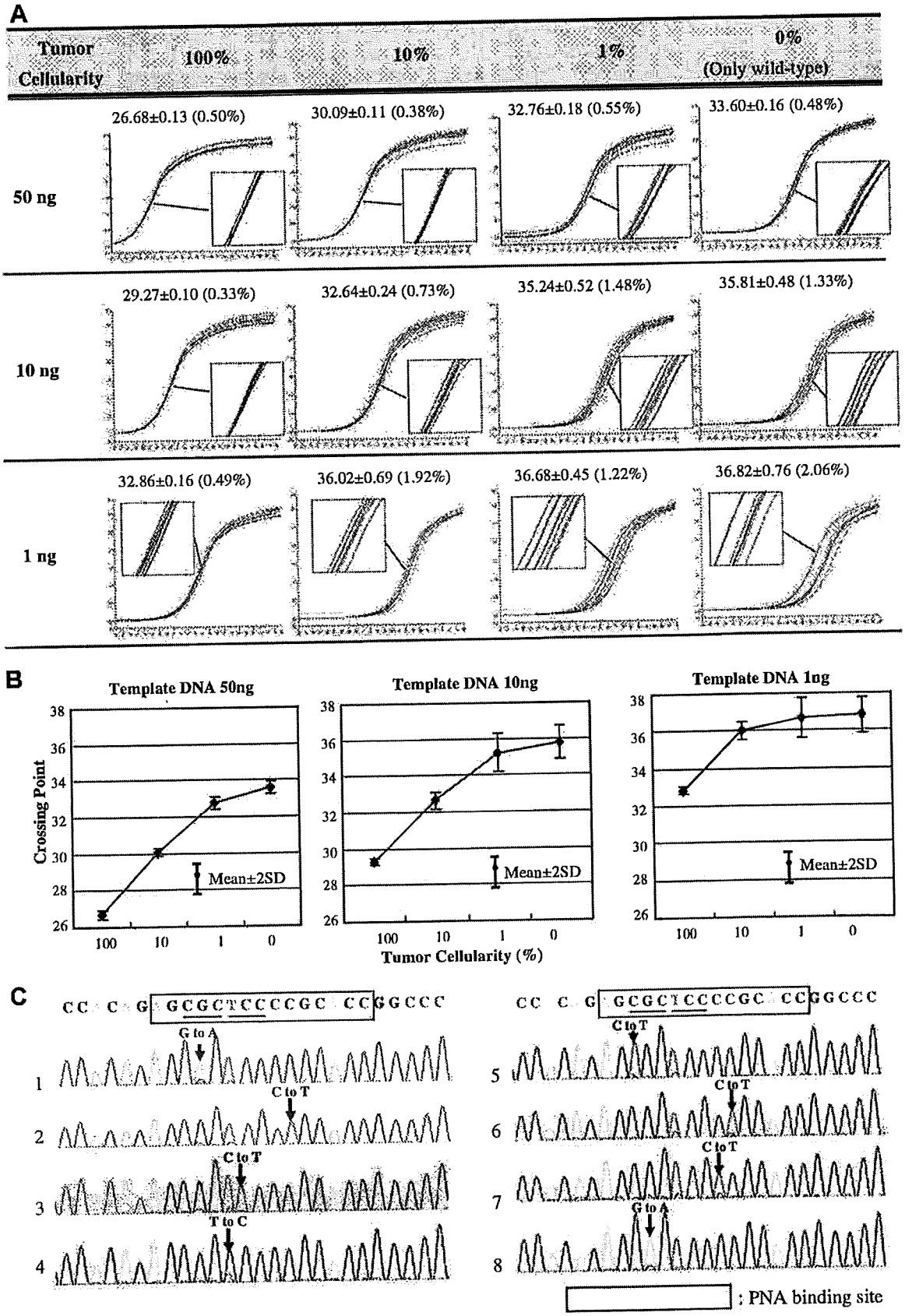

Table 2
Summary of patients' tumor histology, urine cytology and *FGFR3* mutations in tumor tissues and urine sediments

Case No.	Tumor stage/Histological grade	Urine cytology	*FGFR3* mutation status (Tumor cellularity (%))					
			Tumor tissues			Urine sediments		
			Ex 7	Ex 10	Ex 15	Ex 7	Ex 10	Ex 15
1	pTa/G1	Negative	S249C (34.2)	Y375C (100)	WT	S249C (2.4)	WT	WT
2	pTa/G1	Negative	WT	Y375C (100)	WT	WT	Y375C (2.1)	WT
3	pTa/G1	Atypia	S249C (92.4)	WT	WT	S249C (42.1)	WT	WT
4	pTa/G1	Negative	S249C (55.2)	WT	WT	S249C (2.4)	WT	WT
5	pTa/G1	Negative	WT	Y375C (100)	WT	WT	Y375C (100)	WT
6	pTa/G2	Positive	WT	WT	WT	WT	WT	WT
7	pTa/G2	Atypia	S249C (45.0)	WT	WT	S249C (5.5)	WT	WT
8	pTa/G2	Atypia	WT	Y375C (19.0)	WT	WT	Y375C (34.5)	WT
9	pTa/G2	Atypia	WT	Y375C (6.5)	WT	WT	Y375C (3.8)	WT
10	pT1/G2	Negative	S249C (44.1)	WT	WT	WT	WT	WT
11	pT1/G2	Positive	S249C (67.2)	WT	WT	S249C (51.9)	WT	WT
12	pT1/G2	Negative	S249C (34.6)	WT	WT	S249C (6.8)	WT	WT
13	pT1/G3	Negative	S249C (51.6)	WT	WT	S249C (10.5)	WT	WT

Abbreviations: NE, not examined; ND, not determined; WT, wild-type.

uniform regardless of tumor cellularities, while they increased with the reduction of tumor cellularities and were inversely correlated with the amounts of template DNAs in analysis using 1 ng of genomic DNA as the templates (Fig. 3A).

The association between tumor cellularity and CPs was compared in analyses using 50, 10 and 1 ng as template DNAs ($n = 8$) (Fig. 3B). In analysis using 50 ng of genomic DNA as templates, CPs were significantly different among tumor cellularities ranging from 100%, 10%, 1%, and 0%. In contrast, there was no significance of CPs between 1% and 0% in analysis using 10 ng of genomic DNA as templates and between 10% and 1% or less in analysis using 1 ng of genomic DNA as templates. These results indicated that 50 ng of template DNA was required for the reproducible detection of *FGFR3* mutation at a concentration of 1%, i.e., 100-fold excess amount of wild-type DNA.

Sequencing analyses of amplified DNA fragments at various concentrations were performed and those using 1 ng of template DNAs containing only wild-type DNA showed that all samples had point mutations within the PNA binding sites (Fig. 3C). Mutations seemed to occur at random and in different positions from previously reported mutational hotspots, suggesting that these mutations were artifacts induced by PNA-mediated PCR clamping.

Similar approaches were adapted to construct assay protocols to detect mutations in exons 10 and 15. Genomic DNAs extracted from MGHU3 and J82 were diluted with wild-type genomic DNA and prepared as assay standards for exons 10 and 15, respectively, and assay sensitivities for detecting mutations $\geqslant 1\%$ cellularities were validated in control experiments (data not shown).

Detection of FGFR3 mutations in tissues of bladder cancer and corresponding urine sediments

Mutations of *FGFR3* were detected using the above-mentioned assay conditions in tissues and urine sediments from 13 patients with superficial bladder cancer (pTa stage, 9 cases; pT1 stage, 4 cases) and 6 cases of invasive bladder cancer ($\geqslant$pT2 stage, all cases). In analysis of 13 cases with superficial bladder cancer, *FGFR3* mutations and estimated tumor cellularities in exons 7, 10, and 15 were summarized in Table 2. We defined the CP of the assay standard corresponding to 1% cellularity as a minimal detectable dose for *FGFR3* mutations; therefore, a sample showing a CP less than that of 1% standard on each run was determined to be positive for mutation. *FGFR3* mutations in exon 7 were detected in 8 of 13 (61.5%) superficial bladder cancer tissues and sequencing analyses confirmed that all cases harbored S249C mutation in exon 7 of *FGFR3*. The mean tumor cellularity of mutation-positive cases was 53.0% ($n = 8$, range 34.2–92.4%). In the analysis of urine sediments, S249C mutation was detected in 7 out of 8 cases (87.5%) harboring S249C mutation in primary tumors.

Fig. 3. Variations of crossing points (CPs) defined by tumor cellularities and amounts of template DNA. Fifty nanograms, 10 and 1 ng of standard templates with tumor cellularities ranging from 100%, 10%, 1%, and 0% were subjected to PNA-mediated real-time PCR clamping in octaplicated samples ($n = 8$). (A) CPs are indicated as the mean ± SD (CV%) in each run. Observed CV% was maximal in the analysis of 0% standard (only wild-type DNA) using 1 ng of DNA as the template. Insets magnify variations of amplification curves in the log-linear phase. (B) Tumor cellularities and CPs in 50 ng, 10 ng, and 1 ng of the template DNAs were plotted onto a line graph, in which error bars indicate the mean ± 2SD of CPs in each template DNA quantity. (C) Direct sequencing of DNA fragments amplified by PNA-mediated real-time PCR clamping using 1 ng of template containing only wild-type DNA. The uppermost sequence represents the wild-type sequence. The 15-mer sequence surrounded by a rectangle represents the PNA binding site. Codons 248 and 249 are underlined. Sequencing analysis revealed that the 8 amplicons present various types of mutagenized sequences within the PNA binding site. Black arrow indicates the site of point mutation in each sample.

The mean tumor cellularity of urine sediments was 17.4% ($n = 7$, range 2.4–51.9%). Likewise, *FGFR3* mutations in exon 10 were detected in 5 of 13 (38.5%) tumor tissues of superficial bladder cancer and all cases harbored Y375C mutation. In analysis of urine sediments, Y375C mutation was detected in 4 of 5 cases (80%) harboring Y375C mutation in the primary tumor (Table 2). The mean tumor cellularity of mutation-positive cases was 63.1% ($n = 5$, range 6.5–100%) in tumor tissues and 35.1% ($n = 4$, range 2.1–100%) in urine sediments, respectively. No mutations were detected in exon 15 of *FGFR3* either in tissues or urine sediments (Table 2). In the analysis of muscle-invasive bladder cancer ($n = 6$) and 20 urine samples from patients with chronic cystitis, *FGFR3* mutations were not detected either in tumor tissues or urine sediments (data not shown).

Taken together, *FGFR3* mutations in superficial bladder cancer were detected in 12 of 13 (92.3%) tumor tissues and, in analysis of urine sediments, 11 (91.7%, except case no. 10) of 12 cases harboring mutations in primary tumor. One case (no. 1) showed multiple mutations comprising S249C in exon 7 and Y375C in exon 10 in tumor tissue, but only S249C in exon 7 was detected in the urine sample.

Discussion

The present study is the first report of the PNA-mediated real-time PCR clamping technique for detecting *FGFR3* mutation. The assay can be performed by a simple one-step reaction in a single tube using SYBR® green I fluorescent dye. PNA-mediated real-time PCR clamping screens for the presence or absence of mutations; however, PCR products can be easily subjected to direct sequencing to identify the type of mutation.

The amounts of genomic DNA extractable from urine sediments are often trace, particularly in patients during follow-up after TUR or with a small tumor burden; therefore, the method for detecting mutations should be sensitive and specific enough using a small amount of genomic DNA as the template. We validated that this assay enabled reproducible and reliable detection of mutations in a 100-fold excess amount of wild-type DNA using 50 ng of genomic DNA as the template, while it did not work well when the amount of template DNA was less than 10 ng due to the increase of assay CVs. In analysis using 1 ng of genomic DNA as the template, CPs of 1% or 10% standard was indistinguishable from that of 0% standard. CVs of CPs increased in reverse proportion to the decreasing amount of template DNA. In direct sequencing, all PCR products of 0% standard (wild type) amplified from 1 ng of template DNA showed mutations in PNA binding sites (Fig. 3C). Such mutations seemed to occur at random and this phenomenon may account for the increase of CVs of CPs in the experiment using a smaller amount of template DNA. In PCR, changes in the nucleotide sequence are attributable to errors made by DNA polymerase [13]. One nanogram of genomic DNA corresponds to 300 copies of genomic DNA and the length of the PCR product for exon 7 is 182 bps. Numbers of nucleotides in the target DNA are 5.46×10^4 and random errors will not interfere with subsequent analyses in conventional PCR, considering the fidelity (error rate per nucleotide) of *Taq* DNA polymerase is from $\sim 2 \times 10^{-4}$ to $< 1 \times 10^{-5}$ bps, however, PNA bound to wild-type sequences suppressed the amplification of template DNA. As a result, only part of the template DNA was available for PCR, and DNA fragments carrying misincorporated dNTPs would be amplified selectively. This phenomenon seems to be a limiting factor to define assay sensitivity in PNA-mediated real-time PCR clamping.

Thus, we optimized assay conditions to detect *FGFR3* mutations in exons 7, 10, and 15 in a 100-fold excess amount of wild-type DNA. In analysis using clinical samples, *FGFR3* mutations were detected in 92.3% (12/13) of superficial bladder cancer and no mutations were detected in muscle-invasive bladder cancer (0/6) or urine sediments of chronic cystitis (0/20). *FGFR3* mutations were detected in 91.7% (11/12) of urine sediments of which primary tumors presented *FGFR3* mutations. The types of mutations detected in urine sediments coincided with those of tumor tissues, except for one case (Case no. 1) with double mutations in exon 7 and exon 10, with urine sediments showing only a mutation in exon 7 of the FGFR3 gene. Our results suggested that this assay was highly sensitive and specific for detecting *FGFR3* mutations in urine samples.

Sensitive detection of *FGFR3* mutations in urine samples would be beneficial for monitoring tumor recurrence of superficial bladder cancer and would also be suitable as a favorable prognostic indicator in predicting the minimal risk of their progression to muscle-invasive bladder cancer. Further study is ongoing to elucidate the significance of *FGFR3* mutations detectable in urine samples in the clinical management of bladder UCC.

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Bladder Cancer

Weekly Paclitaxel and Carboplatin against Advanced Transitional Cell Cancer after Failure of a Platinum-Based Regimen

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Abstract

Objective: Weekly administration of paclitaxel plus carboplatin is hypothesized to be an effective second-line treatment for advanced transitional cell cancer after failure of platinum-based regimen. In this phase 2 trial, we tested this hypothesis.

Patients and methods: Patients with advanced transitional cell cancer who showed evidence of progressive or recurrent disease after methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) therapy were eligible for this study. Weekly paclitaxel (80 mg/m^2) and carboplatin (AUC 2) were administered on days 1, 8, 15, 22, 29, and 36; the cycle was repeated every 7 wk until disease progression or intolerable toxicity (maximum 18 doses).

Results: Thirty-five patients entered this study. Among the 31 patients who were assessable, 10 had an objective response (overall response rate: 32.3%, 95% confidence interval, 15.8–48.7%). The median progression-free survival (PFS) and median survival times were 3.7 and 7.9 mo, respectively. Among the 22 patients who received prior MVAC therapy for metastatic disease, 36% had an objective response; their median PFS and median survival times were 4.3 and 7.9 mo, respectively; neither survival time significantly differed from the survival time of those who received prior MVAC as adjuvant setting. Toxicities were mild except one toxic death due to neutropenic sepsis.

Conclusions: Weekly paclitaxel plus carboplatin was a manageable, active second-line treatment for advanced transitional cell cancer after failure of platinum-based therapy.

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1. Introduction

Cisplatin-based regimens such as the combination of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC), and the combination of gemcitabine and cisplatin (GC) are considered standard treatment for advanced urothelial cancer [1,2], but there is no standard treatment for patients who fail such a cisplatin-based regimen. Among newer active agents for urothelial cancer, paclitaxel yielded a 42% response rate as first-line therapy [3]. However, against previously treated patients, the response rate was only 10% [4].

Because platinum is the most active agent for urothelial cancer, salvage therapy for advanced urothelial cancer often includes a platinum agent as a component of combination chemotherapy regimens, such as paclitaxel, methotrexate, and cisplatin [5] or gemcitabine, ifosfamide, and cisplatin [6]. These regimens are active not only against platinum-sensitive disease but platinum-resistant disease. Although these combinations yielded a higher response rate, the toxicities they induced were severe, especially in previously treated patients.

Carboplatin is a less nephrotoxic and less emetogenic platinum compound in which a cyclobutanedicarboxylate moiety has been substituted for the two chloride ligands of cisplatin, and it is more suitable for use in renal-impaired or heavily treated patients. Against urothelial cancer, carboplatin has shown modest activity (14% response rate) [7], but whether carboplatin is inferior to cisplatin is unclear, especially when combined with paclitaxel [8,9]. The combination of paclitaxel and carboplatin is a widely used and effective regimen for ovarian cancer and non–small-cell lung cancer. In a phase 3 randomized controlled trial of first-line therapy for advanced urothelial cancer, the patients who received paclitaxel plus carboplatin had a median survival of 13.8 mo, which was similar to the 15.4 mo obtained with MVAC [9]. Although these results must be interpreted with caution because the study failed to reach its accrual goal, the combination of paclitaxel and carboplatin might have significant activity against urothelial cancer with less toxicity.

Carboplatin has been found to have a synergistic effect with paclitaxel on ovarian cancer in vitro [10]. This combination may have activity even in patients previously treated with platinum [10]. Furthermore, weekly administration of paclitaxel versus administration every 3 wk has been reported to have superior activity against metastatic breast cancer, with sustained cumulative exposure and dose-dense drug delivery [11]. Weekly paclitaxel plus carboplatin has been reported to have significant activity against recurrent ovarian cancer [12], advanced non–small-cell lung cancer [13], and advanced breast cancer [14].

Since weekly administration of 135 mg/m^2 of paclitaxel plus the area under the curve (AUC) 2 of carboplatin already has been reported to be intolerable for predominantly chemotherapy-naïve patients with advanced urothelial cancer [15], weekly administration of 80 mg/m^2 of paclitaxel was considered to be more fit for previously treated patients.

On the basis of these data, we designed a phase 2 study of weekly paclitaxel plus carboplatin in patients with advanced urothelial cancer, after failure of a platinum-based regimen.

2. Patients and methods

2.1. *Eligibility and exclusion criteria*

Patients had to be 18 yr of age or older and have histologically proven transitional cell cancer (bladder, renal pelvis, ureter, or urethra) that was not curable by surgery or radiation therapy. Bidimensionally measurable disease documented within 28 d prior to registration was required. Patients had to have progressive or recurrent disease after MVAC therapy. Patients who had undergone prior treatment with adjuvant or neoadjuvant MVAC therapy were also eligible. At least 3 wk had to have elapsed since the completion of preceding chemotherapy or radiotherapy. Patients who had been treated with any taxanes were ineligible. Although an Eastern Cooperative Oncology Group-Performance Status (ECOG-PS) of 0 to 3 had been an eligibility requirement in the early stage of this trial, after the toxic death of one patient with a PS score of 3, we did not accrue patients with this PS score. Adequate organ function with a normal electrocardiogram, absolute granulocyte count of at least 1500/mm^3, platelet count of at least 100,000/mm^3, serum total bilirubin of no more than 1.5 mg/dl, serum transaminase activity of no more than 100 level IU/l, and creatinine level of no more than 2.0 mg/dl were required. Patients with known central nervous system metastasis, active infection, or inadequately controlled diabetes were excluded.

The protocol was approved by the institutional review board of the National Cancer Center Hospital, and all patients provided written informed consent before treatment.

2.2. *Treatment regimen*

Creatinine clearance was estimated by using the Cockcroft-Gault formula. Paclitaxel was administered on an outpatient basis at a dose of 80 mg/m^2 by 1-h infusion followed by carboplatin at AUC of 2 mg·min/ml by 1-h infusion. Dexamethasone 8 mg, ranitidine 50 mg, and chlorpheniramine 10 mg were administered prior to the paclitaxel infusion to prevent a hypersensitivity reaction. Granisetron 3 mg was administered prior to the carboplatin infusion. The paclitaxel followed by carboplatin was administered on days 1, 8, 15, 22, 29, 36, and repeated three times every 7 wk until disease

progression or intolerable toxicity. The next dose was administered only when the absolute granulocyte count was greater than 1000/mm^3, the platelet count greater than 75,000/mm^3, serum transaminase activity of no more than 100 level IU/l, and serum creatinine level of no more than 2.0 mg/dl, and when no grade 2 or higher nonhematologic toxicities except alopecia were observed. The protocol treatment was discontinued if 2 wk elapsed without fulfilling these criteria. Patients were assessed for a response after every six doses during the treatment period and every 2 mo after the completion of 18 doses.

2.3. *Response and toxicity assessment*

Response was assessed according to unidimensional measurements (Response Evaluation Criteria in Solid Tumors criteria), and toxicity was assessed according to the National Cancer Institute Common Toxicity Criteria (NCI CTC), version 2.0. Progression-free survival (PFS) was defined as time from start of therapy to disease progression, death or the most recent follow-up date; overall survival was defined as time from start of the therapy to death or the most recent follow-up date.

2.4. *Statistical analysis*

The primary end point of the trial was the partial plus complete response rate associated with weekly paclitaxel plus carboplatin in patients with bidimensionally measurable metastatic urothelial cancer. The Simon minimax design was used to plan this study on the assumption that the regimen would not be of interest if the true response rate was less than 10%, but that it would be of interest if the response rate was 30% or more. The study had a power of 80% to detect a 30% response rate. Planned accrual was the accrual of 25 eligible patients or expiration of a 2-yr period. Survival curves were estimated by the method of Kaplan and Meier, and univariate time-to-event comparisons were performed with the log-rank test. Responses according to subgroups were compared with the use of the Fisher exact test.

3. Results

3.1. *Patient characteristics*

Between May 2003 and May 2005, 35 patients with advanced transitional cell cancer were entered into this phase 2 study. Because a response was obtained in 32% of the first 25 patients, patient accrual was continued until the end of the planned 2 yr. One patient was ineligible because the patient had not received MVAC as a prior treatment. Three patients were excluded from the final analysis because they received gemcitabine monotherapy before the experimental therapy. Ultimately, 31 patients, 22 men and 9 women, were evaluable for response, toxicity, and survival (Table 1). Their median age was 67 yr (range: 51–80). Twenty-seven patients (87%) had a PS score of 0 or 1, three patients had a PS score of 2, and one patient had a PS score of 3. The site of the primary lesion was the bladder in 45% of the patients. Seventy-one percent of the patients had visceral metastasis. Nine patients (29%) had received prior MVAC as adjuvant or neoadjuvant therapy, and the other 22 patients (71%) had received it for metastatic disease. Platinum-free interval (PFI) was defined as the interval between the final dose of the prior MVAC therapy and the start of weekly paclitaxel plus carboplatin therapy. The median PFI was 4.4 mo (range: 2.5–106). In 18 patients (58%) PFI was less than 6 mo; in the other 13 patients (42%) it was 6 mo or longer. Seven patients had a PFI of more than 1 yr; only one patient had a PFI of more than 2 yr.

Table 1 – Patient characteristics (*N* = 31)

	No. of patients	%
Age, yr		
Median	67	
Range	51–80	
<70	17	55
≥70	14	45
Sex		
Male	22	71
Female	9	29
ECOG-PS		
0 or 1	27	87
2 or 3	4	13
Primary tumor site		
Bladder	14	45
Renal pelvis	9	29
Ureter	7	23
Urethra	1	3
Extent of disease		
Nodal disease only	9	29
Visceral metastasis	22	71
Lung	17	55
Liver	12	39
Bone	5	16
Prior chemotherapy		
MVAC	31	100
Adjuvant therapy	9	29
Against metastatic disease	22	71
Platinum-free interval (PFI)		
<6 mo	18	58
≥6 mo	13	42

ECOG-PS = Eastern Cooperative Oncology Group-Performance Status; MVAC = methotrexate, vinblastine, doxorubicin, and cisplatin.

3.2. *Toxicity*

The median number of doses delivered was 10 (range: 2–18). Hematologic toxicities consisted of ≥ grade 3 granulocytopenia in 18 patients (58%) (grade 3: 39%; grade 4: 19%) and ≥ grade 3 anemia

Table 2 – Toxicity analysis of evaluable 31 patients (National Cancer Institute Common Toxicity Criteria [NCI CTC], version 2.0)

Toxicity	Grade				
	0	1	2	3	4
	n (%)				
Neutropenia	7 (23)	2 (6)	4 (13)	12 (39)	6 (19)
Anemia	0 (0)	9 (29)	11 (35)	6 (19)	5 (16)
Thrombocytopenia	17 (55)	9 (29)	5 (16)	—	—
Febrile neutropenia	28 (90)	—	—	2 (6)	1 (3)
Nausea/vomiting	17 (55)	11 (35)	2 (6)	1 (3)	—
Neuropathy	9 (29)	19 (61)	3 (10)	—	—
Alopecia	7 (23)	7 (23)	17 (55)	—	—
Fatigue	17 (55)	10 (32)	4 (13)	—	—
Diarrhea	26 (84)	4 (13)	1 (3)	—	—

in 11 patients (35%); and no patients developed ≥ grade 3 thrombocytopenia (Table 2). Three patients (10%) experienced ≥ grade 3 febrile neutropenia, and the third patient enrolled whose PS score was 3 died of neutropenic sepsis within 1 mo of the final dose of chemotherapy. Subsequently we did not accrue patients with a PS score of 3.

The most common nonhematologic toxicities were alopecia (grade 1: 23%; grade 2: 55%), neurotoxicity (grade 1: 61%; grade 2: 10%), nausea and vomiting (grade 1: 35%; grade 2: 6%; grade 3: 3%), and diarrhea (grade 1: 13%; grade 2: 3%).

3.3. *Response*

Two of the 31 patients had a complete response, and 8 had a partial response. The overall response rate was 32.3% (95% confidence interval [95%CI], 15.8–48.7%) (Table 3). Among the patients whose PFI was less than 6 mo, 28% (5 of 18) had an objective response, and 38% (5 of 13) of the patients with a PFI of at least 6 mo had an objective response. The difference in the responses between subgroups according to PFI was statistically insignificant. Among the 9 patients who received prior MVAC as adjuvant or neoadjuvant therapy, 2 patients (22%) had an objective response. Among the 22 patients who received prior MVAC for metastatic disease, 8 patients (36%) had an objective response. The difference in the responses between subgroups according to the setting of the MVAC was statistically insignificant. Among the 22 patients who received prior MVAC for metastatic diseases, response rates with regard to response to prior MVAC were also analyzed (Table 4). Although responses were predominantly seen in patients who had responded to prior MVAC, one patient with resistance to prior MVAC responded to weekly paclitaxel plus carboplatin.

3.4. *Survival*

Median follow-up time was 7.8 mo. The median PFS and median survival rates were 3.7 and 7.9 mo, respectively (Fig. 1). Among the patients whose PFI was less than 6 mo, the median PFS and median survival times were 3.7 and 7.8 mo, respectively; neither survival time significantly differed from the survival times of those with PFI of at least 6 mo (median PFS: 3.3 mo; median survival: 12.4 mo). Among the patients who received prior MVAC therapy for metastatic disease, the median PFS and median survival times were 4.3 and 7.9 mo, respectively; neither survival time significantly differed from the survival times of those who received prior MVAC as adjuvant setting (median PFS: 1.6 mo; median survival: 12.4 mo).

Table 3 – Response analysis of evaluable 31 patients

	No. of patients	Response rate	
Overall response	10	32.3% (95%CI, 15.8%–48.7%)	
Complete response	2	6%	
Partial response	8	26%	
Response in PFI < 6 mo	(5/18)	28%	NS
Response in PFI ≥ 6 mo	(5/13)	38%	
Response in prior MVAC as adjuvant therapy	(2/9)	22%	NS
Response in prior MVAC against metastatic disease	(8/22)	36%	
Stable disease	12	39%	
Progressive disease	7	23%	
Not evaluable	4	13%	

95%CI = 95% confidence interval; PFI = platinum-free interval; NS = not significant; MVAC = methotrexate, vinblastine, doxorubicin, and cisplatin.

Table 4 – Response rates according to the response to prior MVAC against metastatic diseases

		No. of patients	Response n (%)
Response to prior MVAC	PR	11	5 (45%)
	SD	3	1 (33%)
	PD	5	0 (0%)
	NE	3	2 (67%)
	Total	22	8 (36%)

MVAC = methotrexate, vinblastine, doxorubicin, and cisplatin; PR = partial remission; SD = stable disease; PD = progressive disease; NE = not evaluable.

4. Discussion

Patients who had received MVAC therapy as prior treatment only in adjuvant or neoadjuvant settings and patients whose disease had progressed after MVAC therapy for metastatic disease were eligible for this phase 2 study. According to Kattan et al's report [16], when a salvage regimen included platinum, time to progression after prior platinum-based therapy, or the PFI, appeared to be important as a basis for interpreting the therapeutic efficacy of salvage treatment as well as whether the prior platinum-based therapy was for metastasis or adjuvant therapy. In this study, we defined PFI as the interval between the final MVAC therapy and the start of weekly paclitaxel plus carboplatin therapy.

Among newer active agents for urothelial cancer, gemcitabine had a 22.5% of response rate as a second-line treatment [17]. The median PFS and median survival times were 3.8 and 5.0 mo, respectively (Table 4). However, since gemcitabine has already become integrated into first-line chemotherapy [1,2], an effective second-line treatment that dose not contain gemcitabine is needed.

Paclitaxel alone yielded a 42% response rate against urothelial cancer in a first-line setting [3] but only a 10% response rate in previously treated patients [4]. Adding ifosfamide to paclitaxel had little effect, and the response rate among 13 patients who had received prior chemotherapy was only 15% [18]. Other promising new active agents are pemetrexed [19] and vinflunine [20] (Table 4), and a randomized phase 3 trial comparing vinflunine with best supportive care after progression following platinum-based chemotherapy is currently under way in Europe.

We found that the weekly paclitaxel plus carboplatin regimen in this study yielded a 32.3% response rate (95%CI, 15.8–48.7%); thus, this second-line treatment appeared to be effective against platinum-pretreated advanced urothelial cancer. This regimen was effective not only in patients with a PFI longer than 6 mo but in patients with a PFI of less than 6 mo, which indicates platinum-resistant disease. Even 28% (5 of 18) of these platinum-resistant patients had an objective response, and their median PFS and median survival times were 3.7 and 7.8 mo, respectively. In addition, 36% (8 of 22) of the patients who received prior MVAC therapy for metastatic disease had an objective response, and their median PFS and median survival times were 4.3 and 7.9 mo, respectively. Responders to weekly paclitaxel plus carboplatin include one patient who did not respond to prior MVAC therapy. These results in patients with platinum-resistant disease appear to be better than the results for weekly paclitaxel described above, which yielded a 10% response rate, and median PFS and median survival times of 2.2 and 7.2 mo, respectively [4]. We think that weekly paclitaxel and carboplatin may exert synergistic activity against advanced urothelial cancer that has

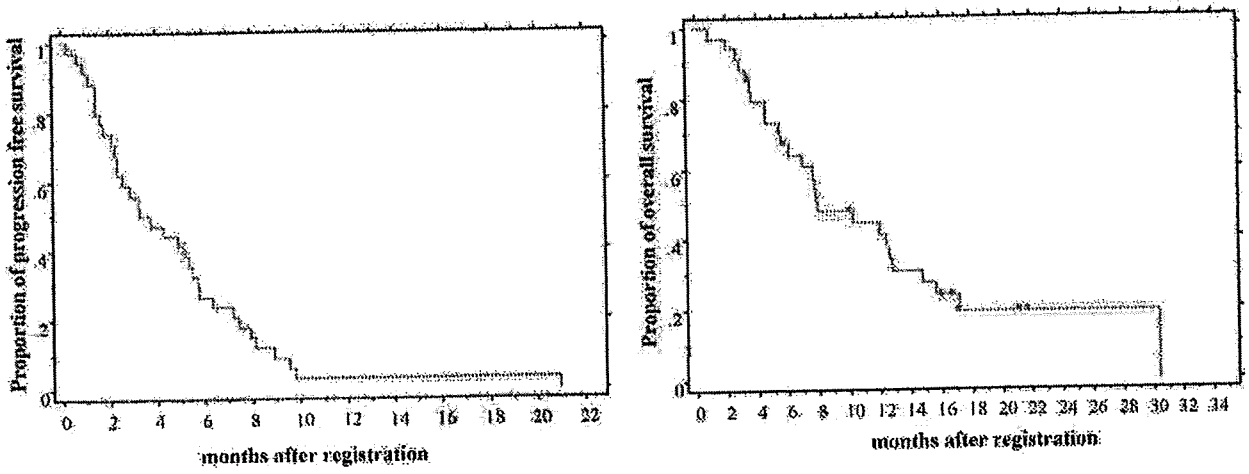

Fig. 1 – Kaplan-Meier curve of progression-free survival and overall survival.

Table 5 – Comparison of recent trials of second-line treatment of advanced urothelial carcinoma except gemcitabine combination regimen

Author, year [reference]	Treatment	No. of patients	Response rate	Median progression-free survival (mo)	Median survival (mo)
Single agent					
Witte, 1997 [22]	Ifosfamide	56	20%	2.2	5.1
McCaffrey, 1997 [23]	Docetaxel	30	13%	NR	9.0
Lorusso, 1998 [17]	Gemcitabine	31	23%	3.8	5.0
Vaughn, 2002 [4]	Paclitaxel (weekly)	31	10%	2.2	7.2
Sweeney, 2006 [19]	Pemetrexed	47	28%	2.9	9.6
Culine, 2006 [20]	Vinflunine	53	18%	3.0	6.6
Combination					
Logothetis, 1991 [24]	Fluorouracil and interferon	30	30%	NR	NR
Tu, 1995 [5]	Paclitaxel, methotrexate, and cisplatin	25	40%	NR	NR
Kattan, 1995 [25]	Ifosfamide, fluorouracil, and folinic acid	15	0%	NR	NR
Otto, 1997 [26]	Paclitaxel, carboplatin, and pertussis vaccine	18	22%	NR	NR
Sweeney, 1999 [18]	Paclitaxel and ifosfamide	26	15%	NR	8.0
De Mulder, 2000 [27]	Fluorouracil, cisplatin, and interferon	43	13%	2.3	4.9
Krege, 2001 [28]	Docetaxel and ifosfamide	20	25%	NR	NR
Di Lorenzo, 2004 [29]	Fluorouracil, folinic acid, and oxaliplatin	16	19%	NR	4.0
Vaishampayan, 2005 [21]	Paclitaxel and carboplatin	44	16%	4.0	6.0
Shinohara, 2006 [30]	Paclitaxel, ifosfamide, and nedaplatin	32	75%	8.0	22
Current series	Paclitaxel and carboplatin (weekly)	31	32%	3.7	7.9

NR = not reported.

failed platinum-containing regimens. Our results are comparable to those obtained with triweekly paclitaxel plus carboplatin in patients previously treated with platinum, which provided a 16% response rate, and median PFS and median survival times of 4 and 6 mo, respectively [21]. Furthermore, our results appear not to be inferior to the results of other second-line treatments that did not contain gemcitabine as a component of combination therapy (Table 5) [22–29]. Recently, Shinohara et al [30] reported a distinguished result for the paclitaxel, ifosfamide, and nedaplatin combination as a second-line treatment, which provided a 75% response rate, and median PFS and median overall survival times of 8 and 22 mo, respectively [30]. These data strengthen our rationale of a combination including paclitaxel and a platimum compound after failure of platinum-based chemotherapy.

Of the 31 patients, 19% experienced grade 4 granulocytopenia, 10% experienced febrile neutropenia, and 1 patient with a poor PS score died of neutropenic sepsis. With the exception of the neutropenic sepsis in the one case of toxic death, the toxicities of weekly paclitaxel plus carboplatin were all manageable. No patient experienced grade ≥3 thrombocytopenia, probably because of the platelet-sparing effect of paclitaxel and carboplatin [31]. In our study, no patient experienced ≥ grade 3 neurotoxicity, and only 10% experienced grade 2 neurotoxicity. Johannsen et al [32] recently reported ≥ grade 3 neurotoxicity in 6% of patients who received first-line weekly paclitaxel (100 mg/m^2) plus carboplatin (AUC 2) for advanced transitional cell carcinoma. In their study, the median number of 12 doses was administered compared with the median number of 10 doses in our study. The less frequent neurotoxicity in our study may be due to the relatively low dose of paclitaxel and the relatively low number of administrations each patient received.

5. Conclusions

Weekly paclitaxel plus carboplatin was a manageable and active second-line treatment for advanced transitional cell cancer after failure of a platinum-based regimen. Paclitaxel plus carboplatin was also effective against platinum-resistant disease, and paclitaxel and carboplatin may act synergistically.

Conflicts of interest

The authors have nothing to disclose.

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Editorial Comment on: Weekly Paclitaxel and Carboplatin against Advanced Transitional Cell Cancer after Failure of a Platinum-Based Regimen

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For many years, the standard first-line chemotherapy in metastatic transitional carcinoma of the urothelium has been the four-drug combination of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC), now replaced in most centers with gemcitabine and cisplatin (GC) with a similar efficacy but with less toxicity [1]. However, no standard has yet been established for second-line treatment. Presently, most interesting single drugs for second-line chemotherapy are gemcitabine, pemetrexed, and vinflunine. In a pooled analysis of seven studies with gemcitabine alone, an overall response rate of 25% and a complete response rate of 9% were achieved [2]. Because the efficacy seems to be independent of whether patients have received prior cisplatin-containing chemotherapy or not, gemcitabine is of potential use as second-line treatment after cisplatin-based chemotherapy not including the drug itself. In the phase 2 study of pemetrexed as second-line chemotherapy by Sweeney et al, an overall response rate of 28% was achieved [3]. This study was, however, not a clean second-line study for metastatic disease because patients with a relapse within 12 mo of adjuvant chemotherapy were also included. Presently, we are awaiting results from the randomized phase 3 study of vinflunine versus best supportive care encompassing a total of 370 patients.

In the phase 2 study by Kouno et al [4], second-line weekly paclitaxel and carboplatin resulted in an overall response rate of 32%. Nine of 31 evaluable patients were included after MVAC as adjuvant treatment. However, the response rate in the remaining patients receiving second-line treatment for metastatic disease was similar to the overall response rate. These results are interesting because paclitaxel is generally considered to be ineffective as second-line treatment following cisplatin-containing chemotherapy. Thus, this combination and schedule of paclitaxel and carboplatin deserves further evaluation.

In conclusion, well-designed studies of second-line chemotherapy for locally advanced or metastatic transitional carcinoma of the urothelium should be given high priority. In that respect, it should, however, be emphasized that patients with a primary good response to combination chemotherapy, such as MVAC or GC, and a long recurrence-free interval generally should be offered reinduction combination chemotherapy and not included in trials with new second-line drugs.

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ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

Patterns of Local Recurrence in Rectal Cancer: A Single-Center Experience

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ABSTRACT A cohort of patients operated at the National Cancer Center Hospital in Tokyo for rectal carcinoma, at or below the peritoneal reflection, was reviewed retrospectively. The purpose was to study the risk factors for local relapse and the patterns of local recurrence. Three hundred fifty-one patients operated between 1993 and 2002 for rectal carcinoma, at or below the peritoneal reflection, were analyzed. One hundred forty-five patients, with preoperatively staged T1 or T2 tumors without suspected lymph nodes, underwent total mesorectal excision (TME). Lateral lymph node dissection (LLND) was performed in suspected T3 or T4 disease, or when positive lymph nodes were seen; 73 patients received unilateral LLND and 133 patients received bilateral LLND. Of the 351 patients 6.6% developed local recurrence after 5 years. TME only resulted in 0.8% 5-year local recurrence. In lymph-node-positive patients, 33% of the unilateral LLND group had local relapse, significantly more ($p = 0.04$) than in the bilateral LLND group with 14% local recurrence. Local recurrence in the lateral, presacral, perineal, and anastomotic subsites was lower in the bilateral LLND group as compared with in the unilateral LLND group. We conclude that, in selected patients, surgery without LLND has a very low local recurrence rate. Bilateral LLND is more effective in reducing the chance of local recurrence than unilateral LLND. Either surgical approach, with or without LLND, requires reliable imaging during work-up.

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For rectal cancer, surgery is the principal treatment in order to cure. Total mesorectal excision (TME) removes the primary tumor with its surrounding mesorectum as an intact package, preventing residual tumor cells in the mesorectum from developing into local recurrence.[1,2] In advanced lesions neoadjuvant (chemo)radiotherapy can downstage tumors, but good surgical quality is still essential in order to achieve total clearance of tumor cells.[3]

The Japanese concept of surgical treatment of rectal cancer has evolved from anatomical studies in which three lymphatic flow routes were identified.[4,5] The upper route is along the superior rectal artery to the inferior mesenteric artery; the lateral route reaches from the middle rectal artery to the internal iliac and obturator basins; and the downward route extends to the inguinal lymph nodes. The upper and lateral routes were shown to be the main two routes of rectal cancer spread, with the peritoneal reflection as the limitation between the two lymphatic areas.[6] Consequently, lateral lymph node dissection (LLND) was developed in Japan in order to resect the tumor with the primary locoregional lymph node basins beyond the mesorectal plane.[7] LLND has resulted in better survival and lower recurrence rates than conventional surgery.[8,9]

A problem is that the lateral lymph node routes are anatomically close to the pelvic autonomic nerve plexus, requiring challenging surgery to preserve these during LLND.[10] In order to prevent damage to autonomic nerves, nowadays case-oriented policy is practised in Japan, adopting LLND only in advanced disease at or below the peritoneal reflection.

The aim of this study is to evaluate the treatment of rectal cancer between 1993 and 2002 at the National Cancer Center Hospital (NCCH), looking at patterns of local recurrence and the risk factors for local recurrence.

PATIENTS AND METHODS

Patients

From 1993 to 2002, 923 patients were operated for confirmed primary adenocarcinoma of the rectum at the National Cancer Center Hospital (NCCH) in Tokyo. Surgery was performed according to the guidelines of the Japanese Research Society for Cancer of the Colon and Rectum.[11,12] The rectum was defined as located below the lower border of the second sacral vertebra. The peritoneal reflection is the most important landmark in defining the location of the tumor, and *low* rectal carcinoma is defined as a tumor of which the major part is located at or below the reflection.[13]

For this analysis the following patients were excluded: metastasis at the time of surgery ($n = 134$) and in situ carcinoma ($n = 22$). Of the remaining 767 patients, only patients with rectal carcinoma at or below the peritoneal refection were selected, resulting in 360 patients.

Neoadjuvant chemotherapy was given to some patients with suspicion of stage T4 disease ($n = 3$) in other hospitals, before referral to the NCCH. Neoadjuvant radiotherapy was not routinely given, so no patients received preoperative radiotherapy. Sometimes in the case of positive lymph nodes, adjuvant radiotherapy ($n = 5$) or chemoradiotherapy ($n = 1$) was given. The nine patients who received neoadjuvant chemotherapy and adjuvant (chemo)radiation were excluded, leaving 351 patients for analysis.

Methods

Until 2002 preoperative evaluation at the NCCH consisted of computed tomography (CT) imaging and endoscopic ultrasonography for all patients. Based on preoperative imaging and intraoperative findings, standard total mesorectal excision (TME) was performed in T1 or T2 stage disease without suspected lymph nodes. Lateral lymph node dissection (LLND) was added to TME in stage T3 or T4 rectal cancer at or below the peritoneal reflection, or when positive mesorectal lymph nodes were suspected. Unilateral LLND was performed when the tumor was located lateral in the low rectum, bilateral LLND when the tumor was located centrally. When the lateral lymph nodes were 1 cm or larger on preoperative imaging or intraoperative findings, bilateral extended lymph node dissection was performed, consisting of dissection of the complete internal iliac artery and the autonomic nerve system. When there was no suspicion on positive lateral lymph nodes, autonomic nerve preservation (ANP) was carried out.

Accurate documentation of lymph node status and localization is obtained because all lymph nodes are harvested and recorded from the fresh specimen. The definition of mesorectal lymph nodes is pararectal location or in the direction of the mesentery. Lateral lymph nodes are located along the iliac or obturator arteries.

Follow-up of all patients consisted of thorax, abdominal, and pelvic CT imaging every 6 months. Median follow-up of patients alive was 7.9 years.

All patients who developed local recurrence, defined as any recurrence of rectal cancer in the lesser pelvis, were identified. Local recurrence was diagnosed clinically, radiologically or histologically.

For all locally recurrent patients the available preoperative images and the images at the time of discovery of the local recurrence were retrieved. A specialized oncologic radiologist (R.G.H.B.-T.) reviewed the images. Examining the images, the site of the local recurrence was determined. The sites were classified into the following regions: lateral, presacral, perineal, anterior or anastomotic. The same borders for the respective sites were used as defined by Roels et al.[14] When no images were available, the location of recurrence was classified using the radiology reports and clinical data. In one patient insufficient information was provided to determine the location of recurrence with certainty.

Statistical Analysis

Statistical analysis was performed using the SPSS package (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL) and R version 2.5.1. *T*-tests and chi-square tests were used to compare individual variables. Survival and cumulative recurrence incidences were estimated using the Kaplan–Meier method. Differences between the groups were assessed using the log-rank test. All *p*-values were two-sided and considered statistically significant at 0.05 or less. For local recurrence, cumulative incidences were calculated accounting for death as competing risk.[15] Similarly, cumulative incidences were calculated for subsite of local recurrence, with death and other types of local recurrence as competing risks, and for cancer-specific survival, with death due to other causes as competing risk. Multivariate analyses of local recurrence and overall survival were performed by first testing the effect of covariates in a univariate Cox regression. Covariates with trend-significant effects (p-value < 0.10) were then selected for multivariate Cox regression. The following variables were studied for local recurrence and overall survival: age, sex, operative procedure, degree of lateral lymphadenectomy, T-stage, mesorectal lymph node N-stage, lateral lymph node positivity, maximum tumor diameter, differentiation, and autonomic nerve preservation.

RESULTS

Clinicopathology

Patient characteristics and treatment details are listed in Table 1. Of the 351 studied patients, 145 had standard TME surgery without LLND, 73 underwent unilateral LLND, and 133 patients received bilateral LLND. LLND was performed in significantly younger patients and more often in combination with a non-sphincter-saving procedure, compared with patients who had not undergone an LLND. The tumors in the LLND patients had higher T- and N-stages and were significantly larger. Comparing the clinicopathological characteristics between the unilateral and the bilateral LLND, no significant differences were found, except that unilateral LLND was more often combined with autonomic nerve preservation (ANP).

Mean lymph node harvest was 21 LNs in standard TME (Table 1). After unilateral LLND the mean number of recovered LNs was 38, and after bilateral LLND this was 45 ($p = 0.004$).

Table 2 shows the outcomes of lymph node involvement for all 351 patients, stratified by T-stage. Overall lymph node involvement was 42%, and lateral lymph node

TABLE 1 Clinicopathological characteristics

	No LLND ($n = 145$)	Unilateral LLND ($n = 73$)	Bilateral LLND ($n = 133$)	p^*	p^{**}
Sex ratio (M:F)	96:49 (66:34)	47:26 (64:36)	86:47 (65:35)	0.95	0.97
Mean age (years)	61	57	57	0.03	0.98
Operation					
Sphincter-saving	112 (77)	36 (49)	63 (47)		
Not sphincter-saving	33 (23)	37 (51)	70 (53)	<0.001	0.79
Adjuvant chemotherapy					
No	139 (96)	67 (92)	121 (91)		
Yes	6 (4)	6 (8)	12 (9)	0.24	0.85
T-stage					
T1	52 (36)	3 (4)	3 (2)		
T2	47 (32)	27 (37)	37 (28)		
T3	46 (32)	40 (55)	83 (62)		
T4	0 (0)	3 (4)	10 (8)	<0.001	0.37
Meso LN positive					
0	102 (70)	44 (60)	64 (48)		
1–3	30 (21)	19 (26)	39 (29)		
>4	13 (9)	10 (14)	30 (23)	0.003	0.28
Lat LN positive					
No	–	62 (85)	109 (82)		
Yes	–	11 (15)	24 (18)	–	0.59
ANP					
No	3 (2)	2 (3)	17 (13)		
Yes	142 (98)	71 (97)	116 (87)	<0.001	0.02
Differentiation					
Well	75 (52)	27 (37)	50 (38)		
Moderate	67 (46)	44 (60)	75 (56)		
Poor	2 (2)	2 (3)	8 (6)	0.18	0.29
Tumor size					
0–4 cm	106 (73)	31 (42)	42 (32)		
>4 cm	39 (27)	42 (58)	91 (68)	<0.001	0.12
Diss. LN (mean)	21	38	45	<0.001	0.004

Values in parentheses are percentages

* p value between no LLND, unilateral LLND, and bilateral LLND

** p value between unilateral LLND and bilateral LLND

Meso mesorectal; *Lat* lateral; *LN* lymph node; *ANP* autonomic nerve preservation

TABLE 2 Lateral lymph node dissection and lymph node status, stratified by T-stage

Stage	LLND		LNI		LNI	LLNI
T1: 58	No LLND	52 (90%)	N0	47	8/58 = 14%	1/58 = 2%
			Upper pos	5		
	LLND	6 (10%)	N0	3		
			Upper pos, lat neg	2		
			Upper neg, lat pos	0		
			Upper pos, lat pos	1		
T2: 111	No LLND	47 (42%)	N0	33	32/111 = 29%	7/111 = 6%
			Upper pos	14		
	LLND	64 (58%)	N0	46		
			Upper pos, lat neg	11		
			Upper neg, lat pos	2		
			Upper pos, lat pos	5		
T3: 169	No LLND	46 (27%)	N0	22	97/169 = 57%	19/169 = 11%
			Upper pos	24		
	LLND	123 (73%)	N0	50		
			Upper pos, lat neg	54		
			Upper neg, lat pos	5		
			Upper pos, lat pos	14		
T4: 14	No LLND	0 (0%)	N0	–	12/14 = 86%	8/14 = 57%
			Upper pos	–		
	LLND	14 (100%)	N0	1		
			Upper pos, lat neg	4		
			Upper neg, lat pos	0		
			Upper pos, lat pos	8		
Total: 351		207/351 = 59%*			149/351 = 42%	35/351 = 10%

LLND lateral lymph node dissection; *LNI* lymph node involvement (upper and lateral lymph nodes); *LLNI* lateral lymph node involvement; Upper, upper lymph nodes; *Lat* lateral lymph nodes; *pos* positive; *neg* negative

* Percentage of patients submitted to LLND

involvement was 10%. Jump metastases (mesorectal lymph nodes negative and lateral lymph nodes positive) occurred in 3% (7/207) of the patients with LLND.

Local Recurrence

At time of last follow-up 23 of the total of 351 patients had developed local recurrence (6.6% 5-year local recurrence rate). In the patients who had not undergone LLND, only one patient (0.8%) had local recurrence at the site of the anastomosis. In the unilateral LLND group, 12 of the 73 patients (5-year 15.4%) had local relapse. This was more than in the bilateral LLND group, with 10 of 133 local recurrences (5-year 8.3%). In N+ patients (Fig. 1), the difference between the uni- and bilateral LLND (32.8% versus 14.2%, respectively) was significant ($p = 0.04$).

In multivariate analysis (Table 3) including uni- and bilateral LLND patients, lateral lymphadenectomy, mesorectal lymph node N-stage, and lateral lymph node positivity were independent risk factors for local recurrence. Compared with patients with bilateral LLND the relative risk for local recurrence was 4.0 for unilateral LLND patients.

Table 4 reports the sites of the local recurrences for the uni- and bilateral LLND groups. The rate of lateral recurrence in the unilateral LLND patients was 5.6%, and in the bilateral LLND patients was 3.3%. It was noticed that the three patients who developed lateral local recurrence on the ipsilateral side after unilateral LLND had lower lymph node harvest (mean 28 LNs) than the patients who developed no lateral recurrence after unilateral LLND (mean 38 LNs). However, the number of patients is too low to draw any firm conclusion from this finding.

Distant Recurrence and Survival

At local recurrence diagnosis 40% of the unilateral LLND patients and 60% of the bilateral LLND patients had distant metastases. One year after local recurrence diagnoses these figures were 70% and 80% in the uni- and bilateral LLND patients, respectively.

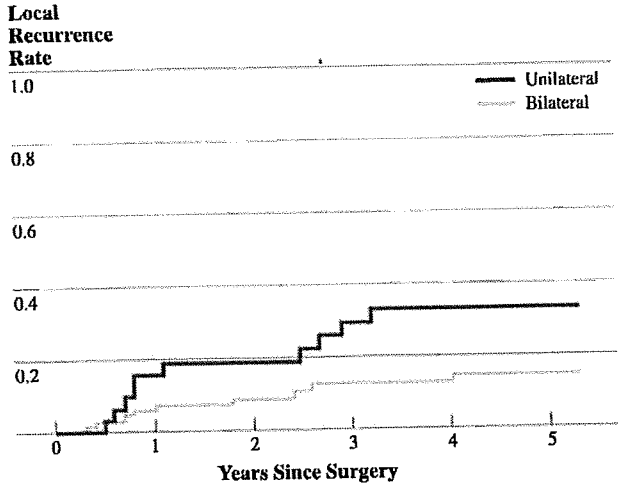

FIG. 1 Local recurrence in N+ patients

Figure 2 shows the survival curves of the TME-only, and uni- and bilateral LLND patients. Overall 5-year survival was 89% for patients who had standard TME. Five-year overall survival in the unilateral LLND group was 78%, which did not differ significantly from the bilateral LLND group (77%) ($p = 0.37$).

The multivariate Cox regression analysis, when including the uni- and bilateral LLND groups, identified T-stage, mesorectal lymph node N-stage and lateral lymph node positivity as independent factors for death risk.

Two years after local recurrence diagnosis 37% of the unilateral LLND patients was still alive, as compared with 60% of the bilateral LLND patients. The number of patients is however too low to conclude significant better survival for bilateral LLND patients.

TABLE 3 Multivariate analysis for local recurrence

Variable	HR	95% CI	*p*
Lateral dissection			0.003
Unilateral	1.00		
Bilateral	0.25	0.10–0.64	
T-stage			0.09
T1 + T2	1.00		
T3 + T4	2.99	0.84–10.73	
N-stage mesorectal LN			0.008
0 pos	1.00		
1–3 pos	2.71	0.75–9.85	
> 4 pos	7.22	2.01–25.94	
Lateral LN status			0.007
Negative	1.00		
Positive	3.53	1.41–8.85	

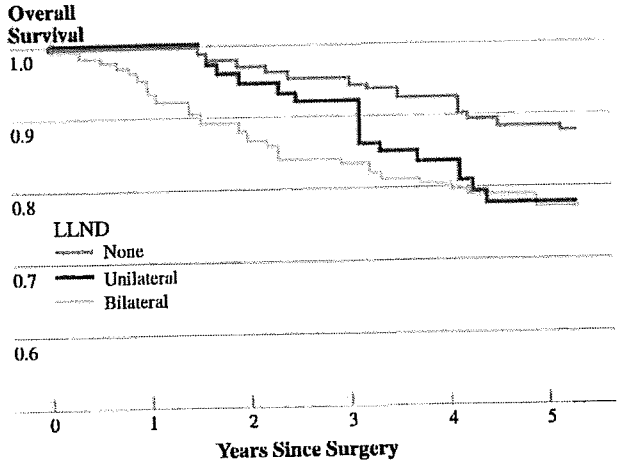

FIG. 2 Overall survival in all patients

TABLE 4 Sites of local recurrence

Site of local recurrence	All patients			Only N+ patients		
	Unilateral LLND ($n = 73$)	Bilateral LLND ($n = 133$)	*p*	Unilateral LLND ($n = 32$)	Bilateral LLND ($n = 74$)	*p*
Lateral	5 (5.6)	4 (3.3)		4 (13.2)	3 (4.6)	
Ipsilateral	*3 (3.4)*			*3 (9.9)*		
Contralateral	*2 (2.2)*			*1 (3.3)*		
Presacral	2 (2.8)	0 (0)		2 (6.7)	0 (0)	
Perineal	2 (2.8)	2 (1.7)		1 (3.1)	2 (3.4)	
Anterior	0 (0)	1 (0.9)		0 (0)	1 (1.8)	
Anastomotic	3 (4.2)	2 (1.6)		3 (9.8)	2 (3.0)	
Unknown	0 (0)	1 (0.8)		0 (0)	1 (1.4)	
Total	12	10		10	9	
5-Year LR rate	15.4%	8.3%	0.06	32.8%	14.2%	0.04

Values in parentheses are the 5-year local recurrence rates per subsite

DISCUSSION

Lateral lymph node dissection (LLND) was introduced in Japan in the 1970s and results in good survival and low local recurrence rates.[7–9] Since approximately 1984 several forms of nerve-sparing techniques, combined with LLND, have been developed. Bilateral and even unilateral complete autonomic nerve preservation (ANP) combined with LLND often maintains urinary function, but reports vary about the results in sexual function.[16–20] In the many decades of LLND surgery in Japan constant evaluation has taken place with the purpose of preventing overtreatment and minimizing morbidity.[21] Nowadays the policy in many Japanese hospitals is highly case-oriented, adapting the degree of surgical resection and ANP to the extent of cancer spread.[22] Whereas in the 1970s and 1980s in the National Cancer Center Hospital (NCCH) in Tokyo the standard procedure was to perform bilateral LLND in case of advanced rectal cancer, lately also unilateral LLND has been performed. The purpose of this study was to evaluate the treatment between 1993 and 2002 at the National Cancer Center Hospital for rectal carcinoma, at or below the peritoneal reflection, looking at the patterns of local recurrence and the risk factors for local recurrence. To our knowledge, there are no published results of unilateral lymph node dissection in rectal carcinoma.

The results of this study show 5-year local recurrence rate of 6.6% in rectal cancer at or below the peritoneal reflection by Japanese surgery. This primarily surgical approach compares favorably with results in Western countries, where neoadjuvant treatment is adopted as the standard in order to reduce local recurrence rates. Therefore, the Japanese concept of removing the lateral basins of lymph nodes spread can be considered successful. However, some questions still remain to be answered. The etiology of locally recurrent disease is not completely understood yet.

This study, although retrospective, provides further evidence of disease outside the TME envelope in higher-stage tumors. Bilateral LLND (5-year local recurrence rate 14%) resulted in better local control than unilateral LLND (5-year LR rate 33%) in N+ patients. Persistent disease in lateral lymph nodes that is left behind may account for some of the local recurrences, as would occur in standard TME surgery. However in that case, it would be expected that most of the recurrences would occur originating in this lateral basin. In this study we noted that only a part of the local recurrences was present in the lateral side walls. Most of the recurrences could not be explained by the anatomical position of the lateral lymph nodes. One can only speculate about other mechanisms of how tumor cells seed into the surgical resection volume. Maybe removal of the lateral lymph nodes also removes (microscopic) tumor cells which are in transit in the lateral lymph flow route, which could otherwise leak back into the surgical wound. This would explain why unilateral dissection is inferior to bilateral dissection, having more local recurrence in also the presacral, perineal, and anastomotic subsite, not only the lateral.

The rationale behind the unilateral LLND is that the contralateral autonomic nervous system stays untouched, decreasing the chance of autonomic nerve injury. Studies report that, after LLND with nerve-sparing surgery, urinary function is maintained. Between 50% and 100% of males are sexually active, however with compromised ejaculation.[16,18,19,23] This is ascribed to traction and injury to nerves during the mobilization and electrocautery required for LLND.[18] Unfortunately we have no data on urinary and sexual function of this cohort, being unable to report on the results after unilateral LLND with nerve preservation. Therefore, the question of whether functional results are truly better remains unanswered.

The tumors of the patients who had TME without LLND were smaller and less advanced compared with those of LLND patients. This better staging is reflected in better survival. That only one patient who had standard TME surgery had local relapse (5-year local recurrence 0.8%) is striking. The selection for low-risk disease by pre- and intraoperative evaluation has obviously been accurate. Interesting however, is that pathology (Tables 1 and 2) showed that about 30% of the patients operated by TME had T3-stage or N-positive disease. Pathology seems to filter out more metastatic lymph nodes than preoperative imaging, but these (micro)metastases obviously have no oncologic consequences. Jump metastases (mesorectal negative, lateral positive) occurred in only 3% of the LLND patients, thus when mesorectal lymph nodes are unsuspected, risk for lateral lymph node recurrence is very low.

Preoperative evaluation in advanced disease is difficult. In this study local recurrence developed on the contralateral side after unilateral lymph node dissection, while these contralateral lymph node metastases were not suspicious on preoperative CT imaging. Meta-analysis report that assessment of lymph node status by CT is unreliable for clinical decision making, because the radiologist can only look at lymph node size.[24,25] Since 2002 in the NCCH magnetic resonance imaging (MRI) has been used, which is reported to be superior to CT because it can rely on additional morphological criteria, such as signal intensity and border contour.[26–28] Furthermore, lymph-node-specific contrast agents or molecular imaging might play a role in detecting micrometastases in the near future.[29]

In the West, (chemo)radiation is used instead of LLND. There are no (randomized) studies comparing preoperative