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# Postoperative recurrence detection using individualized circulating tumor DNA in upper tract urothelial carcinoma

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## Abstract

Biomarkers that could detect the postoperative recurrence of upper tract urothelial carcinoma (UTUC) have not been established. In this prospective study, we aim to evaluate the utility of individualized circulating tumor DNA (ctDNA) monitoring using digital PCR (dPCR) as a tumor recurrence biomarker for UTUC in the perioperative period. Twenty-three patients who underwent radical nephroureterectomy (RNU) were included. In each patient, whole exome sequencing by next-generation sequencing and *TERT* promoter sequencing of tumor DNA were carried out. Case-specific gene mutations were selected from sequencing analysis to examine ctDNA by dPCR analysis. We also prospectively collected plasma and urine ctDNA from each patient. The longitudinal variant allele frequencies of ctDNA during the perioperative period were plotted. Case-specific gene mutations were detected in 22 cases (96%) from ctDNA in the preoperative samples. Frequently detected genes were *TERT* (39%), *FGFR3* (26%), *TP53* (22%), and *HRAS* (13%). In all cases, we obtained plasma and urine samples for 241 time points and undertook individualized ctDNA monitoring for 2 years after RNU. Ten patients with intravesical recurrence had case-specific ctDNA detected in urine at the time of recurrence. The mean lead time of urinary ctDNA in intravesical recurrence was 60 days (range, 0–202 days). Two patients with distal metastasis had case-specific ctDNA in plasma at the time of metastasis. In UTUC, tumor-specific gene mutations can be monitored postoperatively as ctDNA in plasma and urine. Individualized ctDNA might be a minimally invasive biomarker for the early detection of postoperative recurrence.

**Abbreviations:** AC, adjuvant chemotherapy; cfDNA, cell-free DNA; CT, computed tomography; ctDNA, circulating tumor DNA; dPCR, digital PCR; N, lymph node metastasis; NAC, neoadjuvant chemotherapy; NGS, next-generation sequencing; RFS, recurrence-free survival; RNU, radical nephroureterectomy; *TERT*p, *TERT* promoter; UC, urothelial carcinoma; UTUC, upper tract urothelial carcinoma; VAF, variant allele frequency; WES, whole exome sequencing.

KEYWORDS
case-specific mutation, circulating tumor DNA, digital PCR, postoperative monitoring, upper tract urothelial carcinoma

## 1 | INTRODUCTION

Upper tract UC arising from the renal pelvis or ureter is a relatively rare disease, accounting for approximately 5% of UC.[1] The standard treatment of nonmetastatic UTUC patients is radical nephroureterectomy. Upper tract UC is often more advanced at the initial diagnosis, as compared with urothelial bladder carcinoma, and has a high recurrence rate.[2] Common methods for the detection of recurrence are cystoscopy, urine cytology, and CT.[3] However, cystoscopy is invasive, and the sensitivity of urine cytology for UTUC is low.[4] For CT, the sensitivity is low due to the suboptimal detection limit.[5] It is highly important to establish a minimally invasive biomarker with high sensitivity and specificity for recurrence detection in the postoperative period in UTUC.

Postoperative adjuvant therapy is expected to improve the prognosis of locally advanced UTUC.[6] Nivolumab, an anti-PD-1 Ab, showed efficacy in preventing recurrence when given as an adjuvant therapy to patients with UC at a high risk for local and metastatic recurrence after surgery.[7] The phase III POUT trial using adjuvant platinum-based chemotherapy for locally advanced UTUC showed significantly improved disease-free survival among patients.[8] However, these treatments are not effective for all postoperative patients because nivolumab was less effective in UTUC than in bladder cancer and some patients are ineligible for platinum-based chemotherapy due to postoperative renal dysfunction. Therefore, for patients with UTUC, individualized postoperative treatment is desirable.

Circulating tumor DNA is shed by tumor cells into bodily fluids, such as blood and urine. The ctDNA analysis has shown promising results for monitoring recurrence in several cancers.[9] In particular, ctDNA detection using urine samples could be feasible because UC is exposed to urine directly. Several studies have reported the feasibility of ctDNA for UC detection.[10–14] In contrast, only a few studies have reported the utility of ctDNA for UTUC,[15,16] and no study has reported the utility of ctDNA as a tumor biomarker for monitoring UTUC recurrence after surgery. Typical methods for ctDNA analysis are NGS and dPCR. Although NGS can perform a comprehensive gene analysis, it has low sensitivity and is costly; obtaining the results also takes a long time. Conversely, dPCR has high sensitivity and low cost, and obtaining the results takes a short time; however, it can only perform a limited gene analysis, and ctDNA monitoring using existing primer/probe sets can be performed in only limited cases.[16,17] We have recently reported the utility of plasma ctDNA for monitoring of patients with esophageal cancer and established the individualized ctDNA monitoring system to compensate for the weaknesses of NGS and dPCR.[18,19] The aim of this prospective study is to evaluate the utility of individualized ctDNA monitoring using dPCR as tumor recurrence biomarkers for UTUC in the perioperative period.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and clinical samples

This prospective study included 23 patients diagnosed with UTUC who underwent RNU at our institution from January 2019 to December 2020. No patient had other active cancers at the time of enrollment. Patients who underwent NAC or AC were treated. Histological diagnosis was determined by experienced pathologists. Tumor stage and grade were determined according to the 8th edition of the American Joint Committee on Cancer (AJCC) stage classification.[20]

Figure 1A presents a summary of all processes. The DNA from tumor tissues and corresponding PBMCs were collected, and WES and amplicon sequencing for *TERT*p were carried out. Tumor tissues were obtained from fresh frozen tissues of the surgical specimen and PBMCs were obtained from the preoperative blood. Case-specific mutations were selected for monitoring based on the WES results.

Blood and urine samples were collected before and after RNU. The longitudinal VAFs of ctDNA that were analyzed by using dPCR were plotted on a time course along with the diagnostic imaging techniques, such as cystoscopy, urine cytology, and CT. We collected blood and urine samples at 2 days, 1 month, 3 months, and every 3 months thereafter until 2 years after RNU. We undertook cystoscopy, urine cytology, and CT every 3 months on the patients after undergoing RNU (Figure 1B). Cystoscopy was carried out by experienced urologists, and urine cytology was evaluated by specialists. Computed tomography diagnosis was performed by experienced radiologists according to the RECIST criteria.[21]

All patients provided written informed consent, and the study was approved by the Institutional Review Board of our institution in compliance with the guidelines stipulated in the Helsinki Declaration (HG2019-001).

### 2.2 | Extraction of tumor tissue and PBMC DNA

The tumor tissues were obtained immediately after resection and stored at −80°C. Whole blood was collected directly into BD Vacutainer CPT blood collection tubes (Becton, Dickinson and Company) and centrifuged at 1800*g* for 20 min. Buffy coat was collected and centrifuged at 15,000*g* for 3 min. Supernatant was excluded, and the remaining

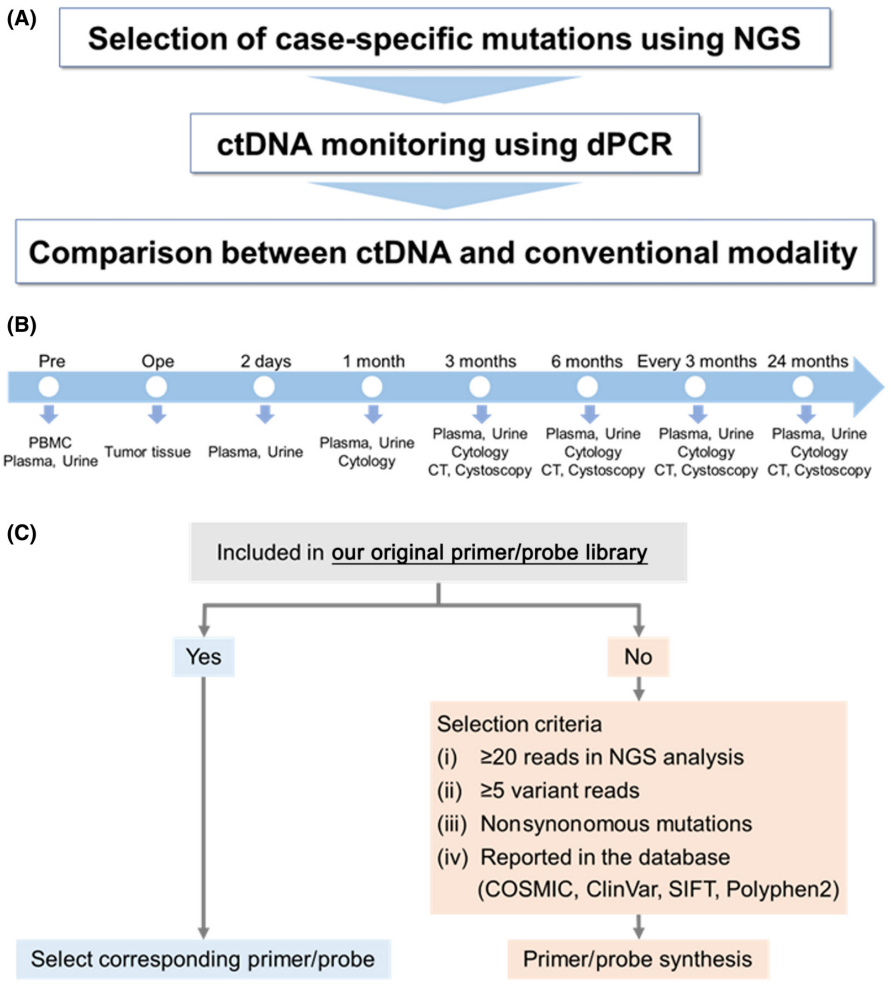


**FIGURE 1** Study design of the present investigation into the detection of postoperative recurrence using individualized circulating tumor DNA (ctDNA) in patients with upper tract urothelial carcinoma. (A) Summary of all processes. (B) Schedule of sample collection and diagnostic techniques. (C) Identification and detection of genes for individualized monitoring using digital PCR (dPCR). CT, computed tomography; ctDNA, circulating tumor DNA; dPCR, digital polymerase chain reaction; NGS, next generation sequencing.

sediment was stored at −80°C within 2 h after collection. Tumor tissue DNA and PBMC DNA were extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol.

### 2.3 | Extraction of plasma and urinary cell-free DNA

Whole blood (8–16 mL) was collected directly into Cell-Free DNA BCT tubes (Streck) and centrifuged twice at 1800 *g* for 20 min. Supernatant (5–10 mL) was stored at −80°C within 7 days after collection. Urine (15 mL) was centrifuged twice at 1800 *g* for 20 min. Supernatant (10 mL) was stored at −80°C within 2 h after collection. Plasma and urinary cfDNA were extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's protocol. Plasma and urinary cfDNA were eluted by 72 μL and 52 μL AVE buffer, respectively.

### 2.4 | Whole exome and amplicon sequencing for *TERT*p

Whole exome sequencing of tumor tissue DNA and corresponding PBMC DNA were undertaken using target capture with SureSelect XT Human All Exon v6 (Agilent Technologies). The raw sequence data were generated using an Illumina HiSeq 2500 platform with a standard 125 bp paired-end read protocol and Illumina NovaSeq 6000 platform with a standard 150 bp paired-end read protocol. We undertook targeted sequencing for *TERT*p by Illumina MiSeq after capturing by SureSelect XT Target Enrichment System (Agilent Technologies).

### 2.5 | Bioinformatics

For our WES data, FASTQ files were generated by bcl2fastq2. Candidate somatic mutations were identified using the Genomon2 pipeline (https://genomon-project.github.io/GenomonPagesR/). The human reference file used was GRCh37/hg19. The candidate mutations in a tumor sample were identified using the following criteria: (i) Fisher's exact test with $p \leq 0.1$; (ii) five or more variant reads in the tumor sample; (iii) VAF in the tumor sample of ≥0.08; and (iv) VAF of the matched normal sample of <0.07, with the exclusion of synonymous single nucleotide variants and known variants listed in NCBI dbSNP build 131.

Targeted sequencing data for *TERT*p were processed in the same way as WES data. We identified the candidate mutations in a tumor sample when those VAFs were ≥0.1.

### 2.6 | Identification and detection of genes for individualized monitoring in urine or blood

For up to two mutations, we selected individualized dPCR probes corresponding to mutations from our originally developed dPCR primer-probe library (https://www.quantdetect.com/product/; Quantdetect Inc.). When three or more mutations were included in our library, we preferentially selected mutations with a higher VAF.

When no appropriate dPCR probes were found in the library, we selected mutations for primer and probe synthesis using the following criteria: (i) ≥20 reads in NGS analysis; (ii) ≥5 variant reads in the tumor sample; (iii) nonsynonymous mutations, and (iv) pathogenic mutations reported in the database, such as COSMIC (https://cancer.sanger.ac.uk/cosmic), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), SIFT (https://sift.bii.a-star.edu.sg/), or Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) (Figure 1C). Without prior knowledge, cases with *TERT*p mutations detected from the tumor tissue by dPCR were used for individualized ctDNA monitoring. When multiple mutations met the criteria, we preferentially selected mutations with a higher VAF.

### 2.7 | Digital PCR analysis for plasma and urinary ctDNA

Before ctDNA monitoring, case-specific primer and probe sets for each mutation were validated using the corresponding primary tumor DNA. Variant allele frequencies were determined using the QuantStudio 3D Digital PCR system (Thermo Fisher Scientific) according to the manufacturer's protocol.

Plasma or urine samples with two or more positive reactions (i.e., dots) for mutant DNA were referred to as ctDNA-positive samples. If only one positive reaction for mutant DNA was detected, samples meeting the following criteria were considered as ctDNA-positive: when more than one positive reaction for mutant DNA could be repeatedly detected upon retesting the same sample; more than one positive reaction for the same mutant DNA was detected in samples collected at similar time points; and more than one positive reaction for another mutant DNA was detected in the same sample. The VAF values were calculated as follows: the fraction of mutant/(mutant + wild) divided by the number of partitions in which either the mutant or WT sequence was detected. If no mutant partition was detected, the case was regarded as negative. These criteria were created in accordance with our previous study.[15]

In addition, we assessed the change rate of VAF between pre-RNU and post-RNU. which were calculated as percentage changes, for example, ([VAF post-RNU / VAF pre-RNU] − 1) × 100.

### 2.8 | Statistical analysis

Statistical analyses were carried out using JMP (SAS Institute) and Prism (GraphPad). Fisher's exact test was used for the analysis of categorical variables, Mann–Whitney *U*-test was used for the analysis of continuous variables, and Kaplan–Meier estimation with log-rank test was used to compare the RFS. The Cox proportional hazards model was used for multivariate analysis. Differences were considered statistically significant when $p < 0.05$.

## 3 | RESULTS

### 3.1 | Patient characteristics

Table 1 presents the characteristics of the patients. The median patient age was 71 years (range, 57–82 years). Of the 23 patients, 13 and 10 patients had renal pelvis and ureter cancers, respectively. Seven patients underwent NAC, and 4 patients underwent AC. Regarding the pathological diagnosis, all patients were pure UC, and did not include histological variants. Eleven and 10 cases were pTa–pT1, and ≥pT2, respectively. Two patients had ypT0 due to NAC. Nineteen patients had pathological high-grade tumor. The median follow-up duration

**TABLE 1** Characteristics of patients with upper tract urothelial carcinoma ($n = 23$)

| | |
|---|---|
| Age (years), median (range) | 71 (57–82) |
| Male sex | 16 (70) |
| Site of primary tumor, renal pelvis /ureter | 13 (57)/10 (43) |
| Smoking history | 11 (48) |
| Chemotherapy, NAC / AC | 7 (30)/4 (17) |
| cT stage, ≤T2 / ≥T3 | 13 (57)/10 (43) |
| cN+ | 5 (22) |
| pT stage, ypT0/pTa–pT1/≥pT2 | 2 (8.7)/11 (48)/10 (43) |
| pN+ | 2 (8.7) |
| Pathological grade, low / high | 4 (17)/19 (83) |
| Postoperative recurrence, bladder / metastasis | 10 (43)/2 (8.7) |
| Observation period (months), median ± SD (range) | 24.7 ± 5.33 (1.37–27.1) |

*Note*: Data are shown as *n* (%), unless otherwise indicated.

Abbreviations: AC, adjuvant chemotherapy; NAC, neoadjuvant chemotherapy.

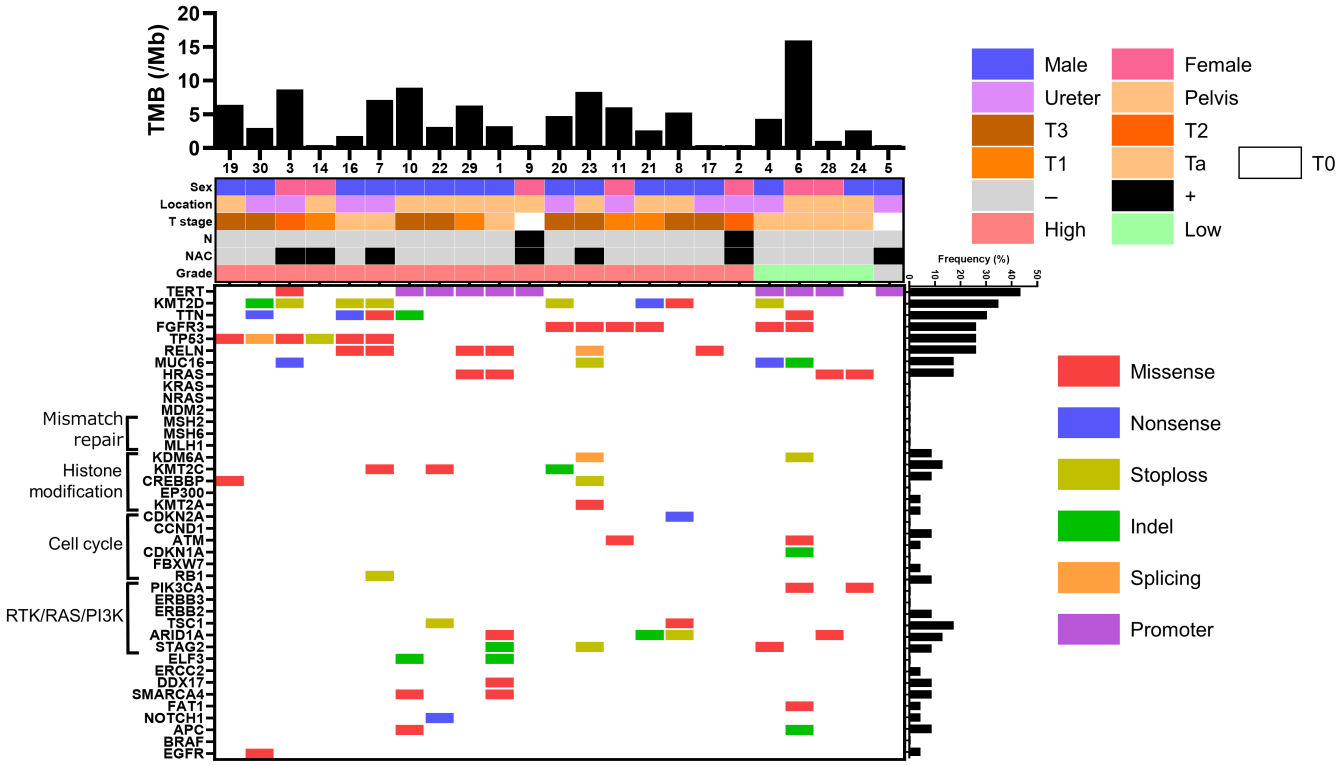


**FIGURE 2** Summary of clinicopathologic characteristics and results of tumor DNA analysis using next-generation sequencing in patients with upper tract urothelial carcinoma. The upper graph shows tumor mutation burden (TMB) for each case, the middle graph shows clinicopathologic characteristics, and the lower graph shows the results of gene mutation analysis and frequency of mutation for each gene. N, lymph node metastasis; NAC, neoadjuvant chemotherapy.

was 24.7 months (range, 1.37–33.6) months. Ten patients (43%) had bladder recurrence and two (8.7%) had metastatic disease; of these patients, one had both bladder recurrence and metastatic disease. The median RFS was 21.9 months (range, 1.37–33.6 months). One patient died due to acute heart failure that was not related to cancer.

## 3.2 | Whole exome and amplicon sequencing for *TERT*p of tumor DNA

The mean depth of WES was 115× (range, 85×–154×), and the mean depth of amplicon sequencing for *TERT*p was 472,903× (range, 386,440×–768,266×). The overall analysis identified an average of 3.23 mutations/Mb (range, 0.02–16.0 mutations/Mb) per patient. One tumor sample (case 6) was hypermutated, with over 10.0 mutations/Mb identified.[22] Frequently mutated genes included *TERT* (43%), *KMT2D* (35%), *TTN* (30%), *FGFR3* (26%), and *TP53* (26%). *TP53* was commonly detected in invasive and high-grade UTUC. Interestingly, mutations in *TP53*, *FGFR3*, and *HRAS* were mutually exclusive (Figure 2).

## 3.3 | Selection of case-specific gene mutations

Case-specific mutations were selected one or two mutations for each case (Table 2). In 19 cases (83%), case-specific mutations were found in our original primer/probe library. We designed novel primers/probes for the remaining four cases. Primer/probe sets were validated by dPCR using each primary tumor with identified target mutations. We frequently selected the following genes: *TERT* (39%), *FGFR3* (26%), *TP53* (22%), and *HRAS* (13%). We frequently selected the following mutations: *FGFR3* p.S249C (26%), *TERTp* C228T (22%), and *TERTp* C250T (17%).

## 3.4 | Monitoring of ctDNA by dPCR during the perioperative period

We obtained plasma and urine samples for 241 time points from 23 cases. In the preoperative samples, 22 cases (96%) had case-specific genetic mutations, whereas urine cytology was positive in 4 (17%) cases. Mutations were detected in 11 cases (48%) in plasma and 20 cases (87%) in urine. Only one case had undetectable mutations, which were ypT0 by NAC. In the preoperative samples, there was no significant relationship between the results of ctDNA and urine cytology (Table S1). In 11 cases with recurrence, 4 (36%) cases had case-specific mutations in plasma and 10 (91%) cases had case-specific mutations. In seven recurrent cases with plasma ctDNA negative in the preoperative samples, we found no significant relationship between ctDNA and patient characteristics (Table S2). One case with recurrence had negative

TABLE 2 Case-specific mutations for each case with upper tract urothelial carcinoma

| | Gene 1 | CDS mutation | AA mutation | Tumor VAF (%) | Gene 2 | CDS mutation | AA mutation | Tumor VAF (%) |
|---|---|---|---|---|---|---|---|---|
| Case 1 | ***TERT*** | **C228T** | - | 59.8 | ***ARID1A*** | **C1897T** | **Q633*** | 48.8 |
| Case 2 | ***TERT*** | **C250T** | - | 1.8 | - | - | - | - |
| Case 3 | *TP53* | T398C | M133T | 81.0 | - | - | - | - |
| Case 4 | ***FGFR3*** | **C746G** | **S249C** | 46.9 | ***TERT*** | **C228T** | - | 45.4 |
| Case 5 | ***TERT*** | **C250T** | - | 19.8 | - | - | - | - |
| Case 6 | ***FGFR3*** | **C746G** | **S249C** | 58.8 | ***PIK3CA*** | **G1633A** | **E545K** | 45.2 |
| Case 7 | ***TP53*** | **G743T** | **R248L** | 62.9 | - | - | - | - |
| Case 8 | ***TERT*** | **C228T** | - | 9.1 | - | - | - | - |
| Case 9 | ***TERT*** | **C250T** | - | 15.0 | - | - | - | - |
| Case 10 | *FGF6* | G395C | G132A | 43.2 | - | - | - | - |
| Case 11 | ***FGFR3*** | **C746G** | **S249C** | 34.0 | - | - | - | - |
| Case 14 | ***TP53*** | **C499T** | **Q167*** | 9.5 | - | - | - | - |
| Case 16 | ***TP53*** | **A536G** | **H179R** | 27.6 | - | - | - | - |
| Case 17 | ***TERT*** | **C250T** | - | 1.3 | - | - | - | - |
| Case 19 | *CDH23* | G2763A | L921= | 5.5 | - | - | - | - |
| Case 20 | ***FGFR3*** | **C746G** | **S249C** | 41.7 | - | - | - | - |
| Case 21 | ***FGFR3*** | **C746G** | **S249C** | 40.0 | - | - | - | - |
| Case 22 | ***TERT*** | **C228T** | - | 22.8 | - | - | - | - |
| Case 23 | ***TERT*** | **C250T** | - | 77.7 | ***FGFR3*** | **C746G** | **S249C** | 71.0 |
| Case 24 | ***HRAS*** | **A182G** | **Q61R** | 94.1 | - | - | - | - |
| Case 28 | ***HRAS*** | **A182G** | **Q61R** | 22.7 | ***TERT*** | **C228T** | - | 17.1 |
| Case 29 | ***HRAS*** | **C181A** | **Q61K** | 66.8 | - | - | - | - |
| Case 30 | *TP53* | 97-2A>G | - | 17.4 | *BAP1* | C2050T | Q684* | 15.7 |

*Note*: Mutations shown in bold were found in our original primer/probe library. * is one of the descriptions of protein variants. * means stop codon.
Abbreviations: AA, amino acid; CDS, coding sequence; VAF, variant allele frequency.

urinary ctDNA in the preoperative sample. This case underwent NAC and showed ypT0.

In the postoperative samples, we undertook individualized ctDNA monitoring using case-specific gene mutations for 2 years after RNU in all cases (Figure 3, Tables S3 and S4). Among 10 cases with bladder recurrence, all were ctDNA positive in urine, whereas cystoscopy, urine cytology, and CT demonstrated recurrence in 10 (100%), 6 (60%), and 3 (30%) cases, respectively, at the time recurrence was diagnosed. All three cases in which bladder recurrence was detected by CT were positive on cytology. The mean lead time of urinary ctDNA in bladder recurrence was 60 days (range, 0–202 days). Three cases (30%) were ctDNA positive in both plasma and urine. Two cases with metastasis were ctDNA positive in plasma, whereas CT showed metastasis in two cases (100%) at the time of metastasis diagnosis. In one case (50%) that experienced synchronous recurrence in the lung and bladder, we detected an increase in VAFs in both plasma and urinary ctDNA. Among 12 cases without recurrence, all were negative in both plasma and urinary ctDNA at 3 months after RNU, when the conventional follow-up techniques were started, and they remained negative during the follow-up period (Figure 4).

Case 9 experienced lymph node metastasis at day 282 after RNU. Plasma ctDNA was positive at the time of recurrence (Figure 5A). Case 17 experienced bladder recurrence on day 311 after RNU. In this case, the VAFs of urinary ctDNA increased 98 days earlier as compared with that at the time of clinical recurrence and decreased quickly after transurethral resection of bladder tumor (Figure 5B). Case 23 underwent NAC before RNU and showed ypT3. However, this case showed no recurrence without AC. In this case, the VAFs of both plasma and urinary ctDNA decreased quickly after RNU and remained negative (Figure 5C). Figures S1–S3 summarize the dynamics of ctDNA during the perioperative period for all cases.

### 3.5 | Prognosis and ctDNA

We investigated several variables that could predict recurrence after surgery. We divided these variables into two groups in terms of clinical recurrence. We compared the change rate of VAFs of plasma and urinary ctDNA from pre-RNU to day 2, 1 month, and 3 months. The change rate of urinary ctDNA between pre-RNU and day 2 after RNU was significantly higher in the recurrence

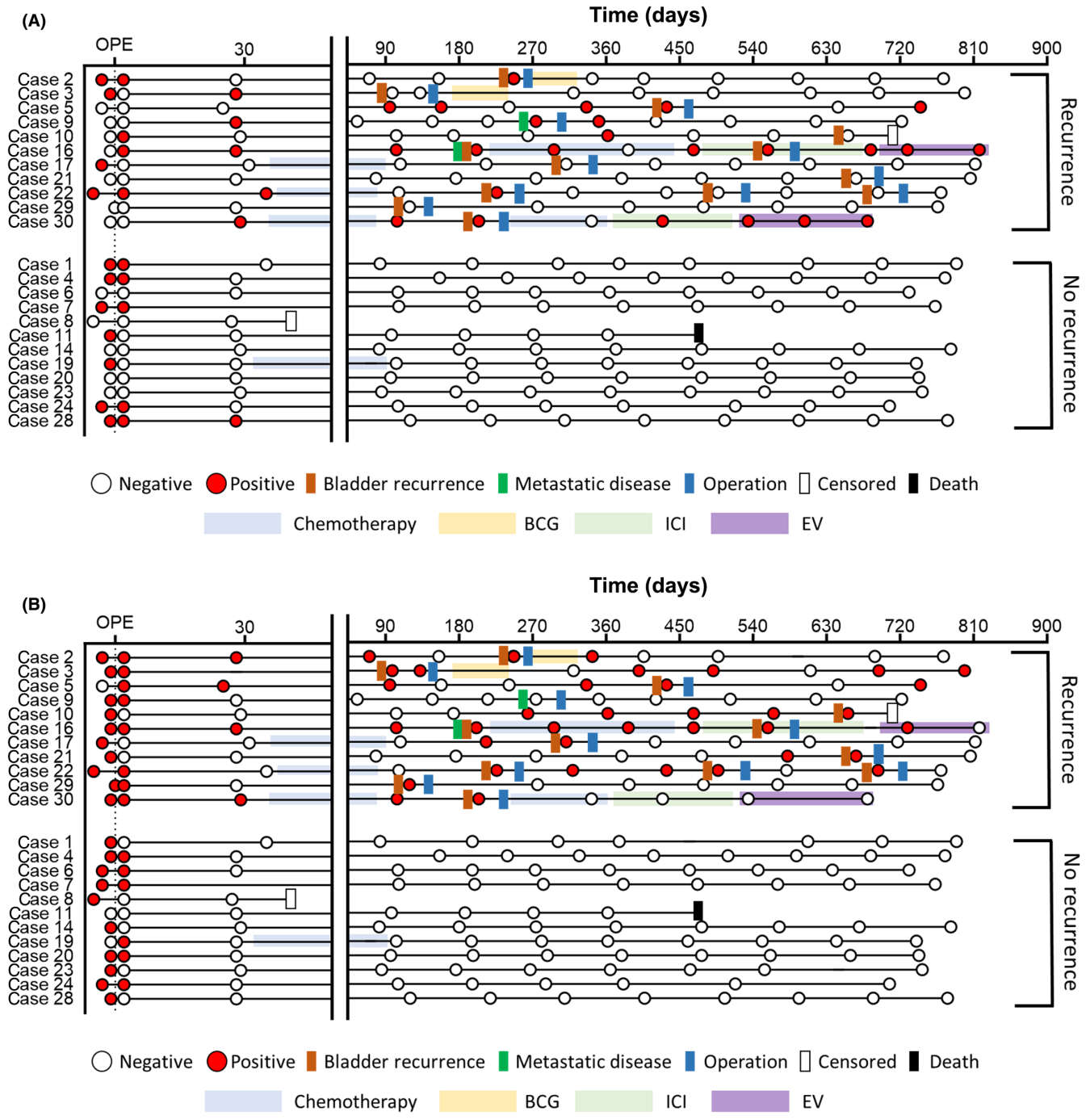


**FIGURE 3** Longitudinal circulating tumor DNA (ctDNA) monitoring using digital PCR in patients with upper tract urothelial carcinoma. The status of ctDNA and clinical information are represented schematically on the horizontal lines. Patients are separated into two groups based on recurrence. (A) Monitoring of plasma ctDNA. (B) Monitoring of urinary ctDNA. BCG, Bacille Calmette–Guerin; EV, enfortumab vedtin; ICI, immune checkpoint inhibitor.

group than in the no-recurrence group (0.00 vs. −99.29, $p = 0.017$) (Figure S4). According to the receiver operating characteristic curve of the change rate of urinary ctDNA data, we defined −95% as the threshold line. We also investigated the clinical, pathological, and ctDNA factors. In the univariate analysis, we identified urine cytology in the preoperative samples and the change rate of urinary ctDNA as prognostic factors associated with short RFS. In the multivariate analysis, only the change rate of urinary ctDNA was identified as a prognostic factor associated with short RFS (Table S5, Figure S5).

## 4 | DISCUSSION

In this prospective study, we evaluated the clinical validity of individualized plasma and urinary ctDNA monitoring as a tumor recurrence

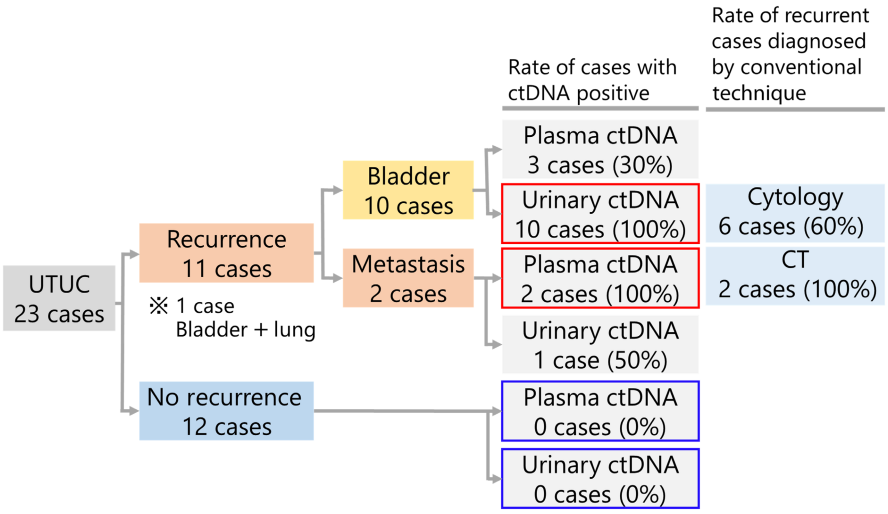


FIGURE 4 Results of circulating tumor DNA (ctDNA) monitoring in patients with upper tract urothelial carcinoma (UTUC). CT, computed tomography.

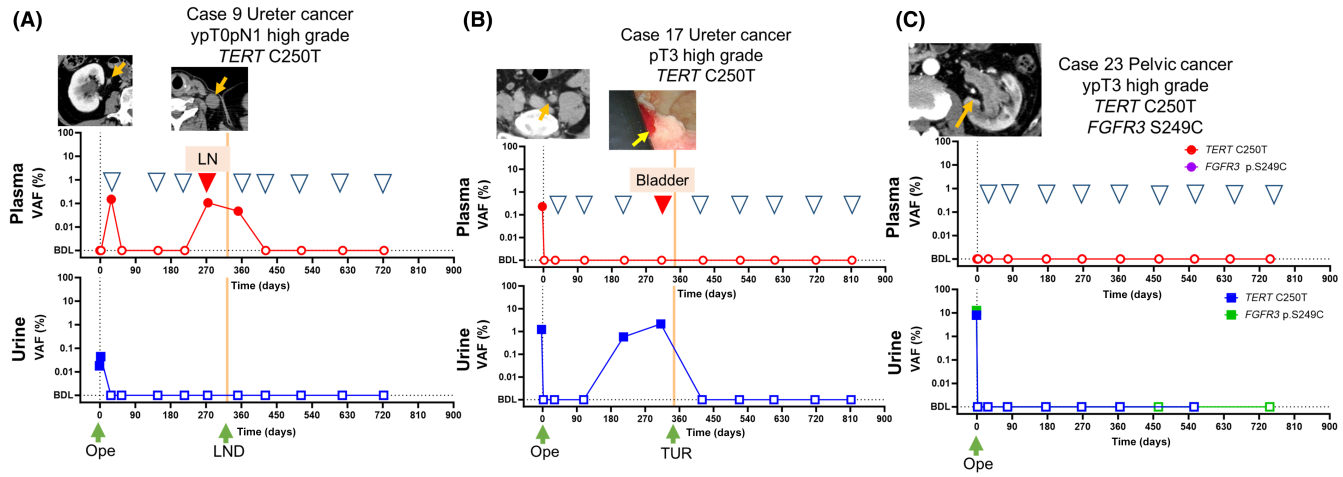


FIGURE 5 Dynamics of circulating tumor DNA (ctDNA) during the perioperative period among patients with upper tract urothelial carcinoma. Filled circles indicate ctDNA positive, and white circles indicate ctDNA negative. White arrowheads indicate no clinical recurrence time points, and red arrowheads indicate clinical recurrence time points. (A) Patient with lymph node metastasis. (B) Patient with bladder recurrence. (C) Patient without recurrence. LN, lymph node; LND, lymph node dissection; Ope, operation; TUR, transurethral resection; VAF, variant allele frequency.

biomarker after surgery for UTUC. For frequent ctDNA monitoring, dPCR is suitable because of its low cost and short turnaround time. In the present study, we first carried out comprehensive gene mutation analysis of tumor tissue and corresponding PBMC by NGS. Second, we compared the tumor mutations with PBMC mutations and selected case-specific genetic mutations for each case to exclude clonal hematopoiesis of indeterminate potential. Third, we undertook ctDNA monitoring using dPCR for tumor-specific gene mutations over time. This prospective study is the first to evaluate the clinical validity of individualized ctDNA monitoring for UTUC using this system. Moreover, by selecting appropriate case-specific mutations, this monitoring system could be used in almost all cases.

Fujii et al.[23] reported that most frequently affected genes in Japanese UTUC cases included *TERT*p, *KMT2D*, *CDKN2A*, *FGFR3*, and *TP53*, whereas mutations in *TP53*/*MDM2*, *FGFR3*, and *RAS* were mutually exclusive. They reported that 95% of UTUC patients had mutations in *TERTp*, *TP53*, *FGFR3*, or *RAS*. In the present study, 20 out of 23 cases (87%) had *TERT*p, *TP53*, *FGFR3*, or *RAS*, and mutations in *TP53*, *FGFR3*, and *HRAS* were mutually exclusive. Fujii et al. also reported that 5.5% of UTUC patients were hypermutated, which is similar to our result (4.3%).

In the present study, 19 of 23 cases (83%) were detected by dPCR probes generated from our original library. For the remaining four cases, the dPCR probes for case-specific mutations were newly designed. These case-specific mutations included those that could not be detected by conventional comprehensive genome profiling tests. Hayashi et al. reported that urinary ctDNA analysis using dPCR of hotspot mutations of *TERTp* (C228T, C250T) and *FGFR3* (S249C) were useful in terms of diagnosis. These hotspot mutations could be detected in 55% of UTUC patients.[16] In the present study, these hotspot mutations could be detected in 12 of 23 cases (52%), and case-specific mutations identified from the tissue in urine could be detected in 20 of 23 cases (87%) before RNU, indicating higher sensitivity than that of the previous

study. We suggest that almost all patients with UTUC can undergo individualized ctDNA monitoring by whole exome sequencing. Case-specific mutations were not detected in one case, which were ypT0 due to NAC. We speculate that the reduction of tumor burden by NAC made the detection of mutations difficult in the preoperative samples.

In this study, all patients with intravesical recurrence after RNU were ctDNA positive in urine, which is a higher proportion of patients than for positive cytology (60%) or CT (30%), and positivity occurred 60 days earlier than with cystoscopy. Moreover, in all metastatic cases, we found ctDNA positivity in plasma at the time of metastasis after RNU. The guidelines of the European Association of Urology recommended stringent follow-up using cystoscopy, urine cytology, and CT after RNU.[3] However, cystoscopy is invasive, and the sensitivities of cytology and CT are low.[4,5] We suggest that the dynamics of ctDNA could detect recurrence earlier and reflect treatment response, which could help to assess the radiographic imaging findings accurately. Case 9 experienced lymph node metastasis after RNU showed plasma ctDNA positivity at the time of recurrence. Although the case-specific mutation in plasma and urine was detected at early time points, these mutations soon after could not be detected just before tumor recurrence was detected. We suggest that, as compared with hematogenous metastasis, lymphatic metastasis could be difficult to detect in ctDNA from plasma and urine samples. We also suggest that relapse-free status could be confirmed by ctDNA negativity. One patient who was indicated for adjuvant nivolumab showed a reduction in VAFs of ctDNA promptly after surgery (case 23). In a phase III trial using adjuvant atezolizumab in UC, the authors reported the utility of ctDNA as the indication of adjuvant therapy.[24] We suggest that the dynamics of ctDNA could be the indication of adjuvant therapy and could help to reduce unnecessary therapy.

Among the 11 recurrent cases, seven had no case-specific mutations in the preoperative plasma samples. After retesting the same sample, these results were reproducible, and case-specific primer and probe sets were validated using tumor DNA. We examined the association between preoperative negative plasma ctDNA and patient characteristics but detected no significant association. However, all cases with <pT2 showed negative preoperative plasma ctDNA. We suggest that cases with early-stage tumors could be difficult to detect in plasma ctDNA. Similarly, one case had no case-specific mutations in either the preoperative plasma or urine samples. This case showed ypT0 due to NAC. The shrinkage of the tumor burden by chemotherapy is considered to make ctDNA from plasma and urine in preoperative samples difficult to detect. Moreover, among seven cases with no case-specific mutations in the preoperative plasma samples, five cases were subsequently positive during postoperative plasma ctDNA monitoring. We suggest that minimal residual disease DNA was shed into blood due to the surgical procedure or that other tumors with the same mutations as the primary tumor recurred postoperatively.

Lughezzani et al.[2] reported several clinical predictive factors of recurrence in UTUC, which included age, smoking, and carcinoma in situ. In the present study, clinicopathologic factors including urine cytology did not show significance in terms of prognosis, whereas a change rate of urinary ctDNA of >−95% was identified as a prognostic factor for poor prognosis. Among all recurrent cases in this study, 91% experienced bladder recurrence. Overall, our study suggests that the change in urinary ctDNA could serve as a useful molecular biomarker of recurrence in UTUC.

The present study has several limitations. First, the number of patients was insufficient to draw definitive conclusions, especially, the number of metastatic cases was small. Second, this was a single-center study, which might have resulted in selection bias. Third, the relationship between case-specific mutations and cancer biology was unclear. Further investigation with an adequate sample size is warranted to confirm our results, especially for metastasis. However, this study was the first to show the clinical validity of plasma and urinary ctDNA by frequent monitoring for the evaluation of therapeutic efficacy and early detection of recurrence in patients with UTUC.

In conclusion, tumor-specific genetic mutations in UTUC can be monitored postoperatively in plasma and urinary ctDNA. Individualized ctDNA might be a minimally invasive biomarker for the early detection of postoperative recurrence.

## AUTHOR CONTRIBUTIONS

**Daichi Tamura:** Conceptualization; data curation; formal analysis; investigation; methodology; resources; supervision; writing – original draft; writing – review and editing. **Masakazu Abe:** Conceptualization; data curation; formal analysis; investigation; methodology; writing – review and editing. **Hayato Hiraki:** Data curation; formal analysis; methodology; resources; writing – review and editing. **Noriyuki Sasaki:** Methodology; resources; writing – review and editing. **Akiko Yashima-Abo:** Methodology; resources; writing – review and editing. **Daiki Ikarashi:** Conceptualization; data curation; formal analysis; investigation; methodology; writing – review and editing. **Renpei Kato:** Conceptualization; data curation; formal analysis; investigation; methodology; writing – review and editing. **Yoichiro Kato:** Conceptualization; data curation; investigation; writing – review and editing. **Shigekatsu Maekawa:** Conceptualization; data curation; formal analysis; investigation; methodology; writing – review and editing. **Mitsugu Kanehira:** Conceptualization; data curation; investigation; writing – review and editing. **Ryo Takata:** Conceptualization; data curation; investigation; writing – review and editing. **Kazuhiro Maejima:** Formal analysis; software; writing – review and editing. **Shota Sasagawa:** Formal analysis; software; writing – review and editing. **Masashi Fujita:** Formal analysis; software; writing – review and editing. **Yutaka Suzuki:** Formal analysis; software; writing – review and editing. **Hidewaki Nakagawa:** Formal analysis; methodology; software; supervision; writing – review and editing. **Takeshi Iwaya:** Methodology; resources; supervision; writing – review and editing. **Satoshi S. Nishizuka:** Conceptualization; data curation; formal analysis; methodology; resources; supervision; writing – review and editing. **Wataru Obara:** Conceptualization; data curation; formal analysis; investigation; methodology; supervision; writing – original draft; writing – review and editing.

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## CONFLICT OF INTEREST STATEMENT

D. Tamura reports grant/research support from Nippon Kayaku, and Quantdetect. M. Abe reports grant/research support from Nippon Kayaku and Quantdetect. H. Hiraki is a consultant of Quantdetect and reports grant/research support from Quantdetect and LSI Medience CO. A. Yashima-Abo reports receiving consultation fees from PCL Japan, Kotobiken Medical Laboratories, LSI Medience, and Quantdetect. H. Nakagawa is an Editorial Board Member of *Cancer Science*. T. Iwaya is a consultant of Quantdetect and reports grant/research support from Nippon Kayaku, Chugai Pharmaceutical, Daiichi Sankyo, and Quantdetect. S. S. Nishizuka reports grant/research support from Taiho Pharmaceuticals, Boehringer-Ingelheim, Geninus, Nippon Gene, LSI Medience, Quantdetect, Iwate Industrial Research Institute, and Thermo Fisher Scientific, honorarium from MSD and Fingal-link, and consultation fees from CLEA Japan, and is a stockholder of Quantdetect. W. Obara reports grant/research support from Astellas Pharmaceuticals, AstraZeneca, Pfizer, Ono Pharmaceuticals, and Taiho Pharmaceuticals. The remaining authors declare no competing interests.

## ETHICS STATEMENT

Approval of the research protocol by an institutional review board: This study was approved by the Institutional Review Board of our institution in compliance with the guidelines stipulated in the Helsinki Declaration (HG2019-001).

Informed consent: All patients provided written informed consent.

Registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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