Circulating tumor cells and cell-free tumor DNA analyses in urothelial cancer using the LiquidBiopsy platform

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1. Introduction

A number of programmed death (PD)-1/PD-1 ligand (PDL-1) inhibitors were recently approved for use in patients with advanced urothelial cancer (UC).\textsuperscript{[1]} However, their effectiveness was reported to be no more than 17\%–26\% despite their high cost. Therefore, the development of companion diagnostic tools to predict the effectiveness of novel therapeutic agents is needed in order to suppress expanding medical expenses. In this context, so-called liquid biopsy for cancer patients using novel technologies is gaining the expectation of application for personalized medicine.

Circulating tumor cells (CTCs) have emerged as important blood-borne biomarkers, which are shed from tumor sites, flow through the blood stream, and may contribute to hematogenous spread. The significance of the CTC count in UC has been reported in relation to recurrence, progression, and prognosis based on studies using the CellSearch\textsuperscript{TM} system, which is the only approach approved by the US Food and Drug Administration.\textsuperscript{[2]} However, its sensitivity in UC is low with a detection rate from 17\% to 23\%. A possible reason may be that the EpCAM-based approach for the enrichment of CTC used in the CellSearch system detects tumor cells with epithelial features and fails to detect subpopulations of CTC with mesenchymal features.\textsuperscript{[3]} Moreover, as CTCs take on phenotypic alteration during treatment,\textsuperscript{[4]} assessment of their qualitative change and molecular profile rather than the CTC number may represent a promising complementary application of CTC analysis.\textsuperscript{[5]}

On the other hand, cell-free DNA (cfDNA) in body fluids is another source for liquid biopsy. Cell-free tumor DNA (ctDNA) is shed into the circulation or urine together with the DNA of normal cells. Despite its small fraction in the circulating total DNA, highly sensitive next generation sequencing (NGS) technique allows capture and analysis of ctDNA, making it a promising biomarker. Potential application of cfDNA analysis in terms of diagnosis, determination of mutational load predictive of immunotherapy, or presence of a target for targeted therapy, and prediction of recurrence or survival has been reported in patients with bladder cancer (BC) at different disease states.\textsuperscript{[6]}

In this study we used the antibody cocktail (EpCAM, EGFR, Her2, and Trop2) that covers cells with epithelial mesenchymal transition (EMT) to improve the efficiency of capturing CTC. Subsequently, we analyzed the mutational status not only on the cellular DNA of CTC but also on the cfDNA from the plasma and urine of UC patients using NGS. Thereby, we attempted to

**Abstract**

**Background:** Emerging data suggested that liquid biopsy such as detection of circulating tumor cells (CTCs) and cell-free tumor DNA analysis augments the management of patients with urothelial cancer (UC). We presented our pilot experience of liquid biopsy using the Ion Torrent platform to detect CTCs and genomic alterations in UC.

**Materials and methods:** Blood or urine samples from 16 patients were subjected to CTC and plasma/urine cell-free tumor DNA isolation for next generation sequencing (NGS) using the Ion S5 system to detect mutations among 50 oncogenes on the Ion AmpliSeq Cancer Hotspot Panel.

**Results:** The Ion Torrent platform detected a higher number of CTCs than those in previous studies using the CellSearch\textsuperscript{TM} system. Overall, mutations were detected in 13/16 (81.3\%) patients with a median number of 18 (range 12–25). NGS isolated 17 hotspot mutations from 11 genes and 41 novel genomic alterations from 24 genes, some of which are supposed to be clinically actionable.

**Conclusions:** The Ion Torrent platform efficiently detected CTCs compared with previous reports. NGS with the present system also allowed for detection of gene alterations which are likely to be therapeutic targets and provided an attractive tool to guide personalized therapy for patients with advanced UC.

**Keywords:** Cell-free DNA; Cell-free tumor DNA; Circulating tumor cell; Next generation sequencing; Urothelial cancer
identify the relevant mutations in order to seek for a possibility of cfDNA as a biomarker for patients with UC.

2. Materials and methods

2.1. Patients and biospecimens

Blood samples were collected from 5 patients with upper tract urothelial cancer (UTUC) and 1 patient with BC for CTC counting, and urine samples were collected from 10 patients with BC for cfDNA analysis.

2.2. CTC counting

CTC collection from blood was performed using the automated analyzer, LiquidBiopsy Platform (Cynvenio Biosystems, CVLB-1-001). That is, 10 mL of whole blood was fixed in the fixative solution using the LiquidBiopsy Blood Collection Kit (ThermoFisher scientific, A28171). A white blood cell (WBC) control was recovered from 400 mL of the original sample. CTCs were enriched as previously described. The cellular component was blocked with FcR block and labeled with a biotinylated antibody cocktail consisting of a combination of anti-EGFR, anti-EpCAM, anti-HER2, and anti-TROP2 (Cynvenio Biosystems) followed by streptavidin beads. The labeled blood was processed in the CTC flow cell on the LiquidBiopsy platform. Captured cells were characterized by evaluating immunofluorescent staining with anti-cytokeratin, anti-CD45, and DAPI by EVOS Floid (Thermo Fisher scientific) and the number of CTCs was counted. Captured cells were recovered by centrifugation to produce an enriched cell pellet. The CTC pellet was digested as previously described and diluted with sample buffer. The AmpliSeq library reagents were directly added to the template and further processed for germline and cfDNA samples.

2.3. Extraction of cfDNA from the plasma and urine

Extraction of cfDNA from the plasma was performed as described in the MagMAX Cell-Free DNA Isolation Kit user guide. Briefly, the plasma was centrifuged to remove any residual blood and cell debris. The Binding Solution/Beads Mix was added to the plasma sample to bind cfDNA to the beads and then placed on the DynaMag Magnet to be pelleted against the magnet. The beads were well washed and resuspended in DynaMag Magnet. The DynaMag Magnet was used to let the beads be pelleted. The purified cfDNA was finally obtained in the supernatant. Urine DNA was extracted similarly to the plasma sample.

2.4. Sequencing with the Ion AmpliSeq cancer hotspot panel

DNA was subjected to library preparation using the Ion AmpliSeq Library Kit 2.0 according to the manufacturer’s instruction. The gene panel, Ion AmpliSeq Cancer Hotspot Panel v2, is designed to amplify 207 amplicons covering 2790 COSMIC (catalogue of somatic mutations in cancer) mutations from 50 oncogenes and tumor suppressor genes. A 60 pmol pooled library was used for template preparation on the Ion Chef system and subsequently sequenced on the Ion S5 system (ThermoFisher scientific) using the Ion 520 chip as per manufacturer’s instruction.

2.5. Post-sequencing data analysis

Data from the Ion S5 runs were processed on the Ion Torrent server using a platform-specific pipeline incorporated in the Torrent Suite v5.8 (ThermoFisher scientific) in order to obtain sequence reads, trim adapter sequences, filter and remove poor signal reads, and assign the reads to a given barcode. The reads were mapped to the hg19 (Homo sapiens) reference genome and adjusted to the specific amplicon target regions of the Ion AmpliSeq Cancer Hotspot Panel v2. Variant call by Variant Caller (v58) plugin was used to obtain a set of default parameters optimized for calling somatic variants.

This study was approved by the Institutional Review Board of Dokkyo Medical University (# R-6-5). Informed consent was obtained from all subjects.

3. Results

3.1. Detection of CTC and mutational status from blood samples

Detectable CTCs were captured from all 6 patients with unresectable UC (Fig. 1). The median number of detectable CTC was 303 (range 11–676) in 10 mL of blood (Table 1). We analyzed the mutation of cancer-related genes on CTC as a control mutation of genes on the WBC. The same analysis was conducted on cfDNA in the plasma to determine how gene mutations on the CTC were reflected, and whether the substitution of cfDNA was clarified. Mutations were detected in all the patients with a median number of 15 (range 12–19). The hotspot mutations, Harvey rat sarcoma viral oncogene homolog (HRAS), isocitrate dehydrogenase 1 (IDH1), KIT proto-oncogene receptor tyrosine kinase (KIT), mutl homolog 1 (MLH1), platelet derived growth factor receptor alpha (PDGFRα), phosphatase, and tensin homolog (PTEN) were detected in common across WBC, CTC, and cfDNA, while hotspot mutations in tumor protein p53 (TP53) and fibroblast growth factor receptor 3 (FGFR3) were only detected from cfDNA in 2 patients. That is, mutation profiles on CTC and cfDNA were similar in the majority of cases (4/6) despite some undetectable alterations on CTC. Subsequently, plasma cfDNA NGS isolated 9 hotspot mutations from 8 genes in all 6 patients and 24 novel genomic alterations from 18 genes in 5 patients, anaplastic lymphoma kinase (ALK), adenosomatous polyposis coli (APC), ATM serine/threonine kinase (ATM), colony-stimulating factor 1 receptor (CSF1R), epidermal growth factor receptor (EGFR), Erb-b4 receptor tyrosine kinase (ERBB4), FGFR1, FGFR3, Fms related tyrosine kinase 3 (FLT3), Janus kinase 3 (JAK3), kinase insert domain receptor alpha (KDR), PDGFR3, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), rearranged during transfection (RET), smoothened, frizzled class receptor (SMO), serine threonine kinase 11 (STK11), TP53, and von-Hippel Lindau tumor suppressor (VHL) (Table 2). The identified 9 hotspot mutations were all single nucleotide variants (SNV). Most of the novel mutations were SNVs (20) and insertion (2), deletion (1), and multiple nucleotide polymorphisms (MNP) (1) and were observed in a small number of cases.

3.2. Analysis of the genetic mutations in cfDNA from urine samples

Using two urine samples from patients with UC, we first compared the number of genetic mutations in the urine supernatant and precipitate to determine which was a better source to detect mutations. More mutant DNAs were found in the supernatant than in the precipitate (Table 3). Thus, we decided to use the urine supernatant in the subsequent analysis. The mutational analysis was performed in cfDNA of the urine
Figure 1. Image of captured CTC from a UC patient labelled by immunofluorescent staining with anti-cytokeratin, anti-CD45, and DAPI. CTC = circulating tumor cell.

Table 1

Patient characteristics and result of CTC isolation, sequence, and variant analysis.

| Age, yr | Sex | UC type | TNM   | Metastatic sites | Prior chemotherapy | Source | Cell number | Total no. variants | Hotspot | Gene       |
|---------|-----|---------|-------|------------------|--------------------|--------|-------------|--------------------|---------|------------|
| 73      | Female | UTUC   | T3N2M0| Lymph node       | Yes                | CTC    | 676         | 17                 | 1       | KIT        |
|         |       |        |       |                  |                    | WBC    | 2816        | 15                 | 1       | KIT        |
|         |       |        |       |                  |                    | cDNA   | —           | 13                 | 1       | KIT        |
| 76      | Female | UTUC   | T3N2M1| Lung             | Yes                | CTC    | 78          | 15                 | 2       | IDH1, PTEN |
|         |       |        |       |                  |                    | WBC    | 1180        | 14                 | 2       | IDH1, PTEN |
|         |       |        |       |                  |                    | cDNA   | —           | 14                 | 2       | IDH1, PTEN |
| 74      | Male   | BC     | T3N1M1| Lymph node       | Yes                | CTC    | 11          | 13                 | 1       | MLH1       |
|         |       |        |       |                  |                    | WBC    | 1184        | 14                 | 1       | MLH1       |
|         |       |        |       |                  |                    | cDNA   | —           | 16                 | 1       | MLH1       |
| 77      | Female | UTUC   | T3N2M1| Lung             | Yes                | CTC    | 342         | 16                 | 0       | —          |
|         |       |        |       |                  |                    | WBC    | 1696        | 16                 | 0       | —          |
|         |       |        |       |                  |                    | cDNA   | —           | 18                 | 1       | —          |
| 72      | Male   | UTUC   | T3N2M1| Bone             | no                 | CTC    | 641         | 13                 | 2       | PDGFRA, HRAS|
|         |       |        |       |                  |                    | WBC    | 1368        | 15                 | 2       | PDGFRA, HRAS|
|         |       |        |       |                  |                    | cDNA   | —           | 19                 | 3       | PDGFRA, HRAS, FGFR3 |
| 79      | Male   | UTUC   | T4N1M1| Lymph node       | no                 | CTC    | 265         | 12                 | 1       | KIT        |
|         |       |        |       |                  |                    | WBC    | 1968        | 12                 | 1       | KIT        |
|         |       |        |       |                  |                    | cDNA   | —           | 13                 | 1       | KIT        |

BC = bladder cancer; UTUC = upper tract urothelial cancer.
supernatant from 8 patients with BC who did not undergo cystectomy (Table 4). Successful cfDNA detection was performed in 5/8 (62.5%) cases with a median number of mutations of 18 (range 13–22). The clinical characteristics of ≥T3 or presence of metastasis were associated with successful detection. Twelve hotspot mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A), HRAS, mesenchymal epithelial transition receptor tyrosine kinase (MET), MLH1, PDGFRa, PIK3CA, STK11, and TP53 were observed in 4 patients. A total of 33 novel mutations were found from 20 genes in 5 patients, ALK, APC, CDKN2A, CSF1R, EGFR, ERBB4, FGFR1, FGFR3, FLT3, JAK3, KDR, MET, notch receptor 1 (NOTCH1), PDGFRa, PIK3CA, RET, retinoblastoma 1 (RB1), SMAD family member 4 (SMAD4), STK11, and TP53 (Table 2). Again, the identified 12 hotspot mutations were all SNV. Most of the novel mutations detected were SNV (26) and exceptionally MNP (2), insertion (1), and deletion (1) were also observed.

4. Discussion

In this study, we compared NGS data sets from three distinct patient-matched samples types (WBC, CTC, and cfDNA). A common amplicon based resequencing panel (Ampliseq v2 HotSpot panel) was used for all template types in a NGS pipeline using identical variant analysis. The nature of cancer cells is different depending on the treatment, time lapse, or metastatic sites. Various clinical factors and histological examination of surgical specimens and biopsy tissue provide prognostic information, but hardly predict the efficacy of therapeutic drugs. However, liquid biopsy along with the emergence of NGS made it possible by obtaining all the cancer cells at each time point. NGS based analysis has made a great contribution to the characterization of somatic mutations in cancer genomes and helps guide diagnosis and selection of therapy. Recently, it was reported that androgen receptor splice variant 7 (AR-V7) detection from CTC was associated with worse outcomes for patients with castration-resistant prostate cancer treated with novel androgen receptor-signaling inhibitors such as abiraterone and enzalutamide, but not taxane chemotherapies.[8] Such a breakthrough in cancer therapy has dramatically changed the treatment recommendation in the various guidelines based on previous large-scale clinical trials to individualized treatment. Likewise, prompt development of companion diagnostic biomarkers in UC is also awaited.

This study explored the usefulness of the LiquidBiopsy Platform for CTC and cfDNA analysis in the diagnostic assessment of UC. NGS was performed using the Ion S5 system, in which the Ion chef was used to automate template preparation, enrichment, and chip loading. Preloaded reagent cartridge and on-board Torrent suite software reduces the initialization (10 minutes), sequencing (2.5 hours), and analysis time (2–4 hours). Compared with the commercially available NGS platforms such as Guardant 360 and FoundationOne™, the former which is excellent for detection of specific mutation from cfDNA and the latter which is specially applied to comprehensive mutational analysis from tissue samples, Ion Torrent provides expansive application to CTC, cfDNA, and tissues along with customized PCR primers (Table 5).[9,10]

The number of CTC in patients with metastatic UC was much higher than those counted by the CellSearch system in previous reports (Table 6).[11–15] The tumor specific EpCAM antibody whose expression is often reduced or lost in highly malignant tumors with EMT is used in the CellSearch system to capture CTC.

### Table 2
Number of the identified gene alterations in plasma and urine cfDNA.

| Genes  | Plasma cfDNA |    | Urine cfDNA |    |
|--------|--------------|----|-------------|----|
|        | Novel | Hotspot | Novel | Hotspot |
| ALK    | 2     | 3     | 1     | 1     |
| APC    | 1     | 2     | 1     | 2     |
| ATM    | 1     | 1     | 1     | 1     |
| CDKN2A | 1     | 1     | 1     | 1     |
| CSF1R  | 1     | 1     | 1     | 1     |
| EGFR   | 1     | 1     | 1     | 1     |
| ERBB4  | 1     | 1     | 1     | 1     |
| FGFR1  | 1     | 1     | 1     | 1     |
| FGFR3  | 2     | 1     | 1     | 1     |
| FLT3   | 1     | 1     | 1     | 1     |
| HRAS   | 1     | 1     | 1     | 1     |
| IDH1   | 1     | 1     | 1     | 1     |
| JAK3   | 1     | 1     | 1     | 1     |
| KDR    | 5     | 6     | 6     | 6     |
| KIT    | 2     | 2     | 2     | 2     |
| MET    | 1     | 1     | 1     | 1     |
| MLH1   | 1     | 1     | 1     | 1     |
| NTH1   | 1     | 1     | 1     | 1     |
| PDGFRa | 1     | 1     | 1     | 1     |
| PIK3CA | 1     | 1     | 1     | 1     |
| PTEN   | 1     | 1     | 1     | 1     |
| RB1    | 1     | 1     | 1     | 1     |
| RET    | 1     | 1     | 1     | 1     |
| SMAD4  | 2     | 2     | 2     | 2     |
| SM0    | 1     | 1     | 1     | 1     |
| STK11  | 1     | 1     | 1     | 1     |
| TP53   | 1     | 1     | 1     | 1     |
| VHL    | 1     | 1     | 1     | 1     |
| Total  | 24    | 9     | 33    | 12    |

cfDNA = cell-free DNA.

### Table 3
Cell-free DNA isolation from supernatant and precipitate of urine sample.

| Age | Sex | Stage | Source  | DNA, ng/uL | Variants | Hotspot |
|-----|-----|-------|---------|------------|----------|---------|
| 73  | Male | IV    | Supernatant | 56.4 | 20 | 2 |
| 68  | Male | IV    | Precipitate | 127 | 16 | 2 |
| 73  | Male | IV    | Supernatant | 2.88 | 25 | 1 |
| 68  | Male | IV    | Precipitate | 1.8 | 15 | 1 |
Thus, such a higher CTC count obtained in the present study may be due to the majority of patients having UTUC and the use of the Ion Torrent LiquidBiopsy Platform, in which an antibody cocktail is used to capture CTC. The antibody cocktail includes not only EpCAM but TROP2 and Her2 which are expressed in EMT induction and EGFR whose overexpression is associated with poor prognosis in order to improve the efficiency of CTC collection.[16]

If a higher number of CTC is a feature of UTUC, it may explain its poorer prognosis compared with BC and be a promising biomarker for patients with UTUC.[17] This should be further investigated in a larger cohort of patients with UTUC.

The current comprehensive genomic profiling using the NGS platform has facilitated identification of potential targets within a mutational landscape of tumors, which is an initial step for establishing precision cancer treatment. The Cancer Genome Atlas (TCGA) Project pioneeringly provided important insight into the genomics and biology of UC. The study identified potential therapeutic targets in 69% of the cases, including 42% with targets in the PI3K/AKT/mTOR pathway and 45% with targets (including ERBB2) in the RTK/MAPK pathway in tumor tissues from 131 cases of BC.[18] The subsequent TCGA study analyzed a large number of samples from 412 patients with muscle invasive BC and identified frequent somatic alterations in p53/cell cycle pathways (89%), the RTK/RAS/PI3K pathway (71%), and epigenetic mutations in chromatin-modifying or chromatin-regulatory genes (78%).[19] Ross et al. identified at least 1 clinically relevant genomic alteration in 93% of the tumor tissue from 295 patients with advanced BC. The most common clinically relevant genomic alterations involved CDKN2A (34%), FGFR3 (21%), PIK3CA (20%), and ERBB2 (17%).[20]

cfDNA in blood plasma, derived from cancer cell death and actively released DNA, has been well investigated in various solid tumors.[21]

cDNA NGS is an ideal platform for upfront and serial testing of disease progression in patients with advanced cancer because of its easy and safe sample collection, ability to capture tumor heterogeneity, with lower cost and invasiveness compared with tissue NGS that requires repeated tissue biopsy.

### Table 4

| Age | Sex | TNM | Metastatic sites | Prior TURB | Prior chemotherapy | Gross hematuria | Urinalysis | CTC, ng/mL | Variants | Hotspot Gene |
|-----|-----|-----|------------------|------------|-------------------|----------------|------------|------------|----------|--------------|
| 73  | Male| TaN0M0| Yes | No | No | Normal | II | 1.18 | 0 | 0 |
| 76  | Male| T2N0M0| Yes | No | Yes | Hematuria | II | 0.753 | 0 | 0 |
| 73  | Male| T2N1M0| Lymphnode | Yes | No | Yes | Normal | III | 2.06 | 1 | 0 |
| 71  | Male| T2N2M1| Bone | Yes | No | No | Hematoparyia | III | 1.33 | 22 | 5 |
| 77  | Male| T3N0M0| Yes | Yes | No | Normal | III | 4.96 | 22 | 4 |
| 57  | Female| T3N0M0| Yes | Yes | No | Hematoparyia | NA | 6.71 | 15 | 1 |
| 53  | Male| T3N0M0| No | Yes | Yes | Hematoparyia | III | 13.5 | 18 | 3 |
| 40  | Male| T3N1M1| Lung, bone, brain, lymphnode | Yes | Yes | No | Normal | III | 3.32 | 13 | 0 |

CTC = circulating tumor cell; cfDNA = cell-free DNA; TURB = transurethral resection of the bladder.

### Table 5

| Platform | Ion Torrent | Guadant 360 | FoundationOne |
|----------|-------------|-------------|--------------|
| Input DNA | CTC, cfDNA, tissue | cfDNA | Tissue |
| Molecular DNA barcode | Available | Available | Available |
| Limit of detection | 0.1–5% | 0.40% | 1.5–5.9% |
| Target | 50–500 genes | 73 genes | 324 genes |

1. [https://www.thermofisher.com/jp/en/home/clinical/preclinical-companion-diagnostic-development/oncomine-oncology.html](https://www.thermofisher.com/jp/en/home/clinical/preclinical-companion-diagnostic-development/oncomine-oncology.html).
2. [https://www.ncc.go.jp/en/information/press_release/20180313/index.html](https://www.ncc.go.jp/en/information/press_release/20180313/index.html).
3. [https://www.accessdata.fda.gov/cdrh_docs/pdf17/P170019C.pdf](https://www.accessdata.fda.gov/cdrh_docs/pdf17/P170019C.pdf).

NGS = next generation sequencing; CTC = circulating tumor cell; cfDNA = cell-free DNA.

Thus, such a higher CTC count obtained in the present study may be due to the majority of patients having UTUC and the use of the Ion Torrent LiquidBiopsy Platform, in which an antibody cocktail is used to capture CTC. The antibody cocktail includes not only EpCAM but TROP2 and Her2 which are expressed in EMT induction and EGFR whose overexpression is associated with poor prognosis in order to improve the efficiency of CTC collection.[16] If a higher number of CTC is a feature of UTUC, it may explain its poorer prognosis compared with BC and be a promising biomarker for patients with UTUC.[17] This should be further investigated in a larger cohort of patients with UTUC.

### Table 6

| Reference number | Site of UC | Number of CTC positive patients (%) | Number of cases with N+ | Number of cases with M1 | Median CTC |
|------------------|-----------|------------------------------------|------------------------|------------------------|------------|
| [11]             | BC        | 42 (22.3)                          | 17                     | 0                      | 1         |
| [12]             | BC        | 12 (27%)                           | 3                      | 7                      | 2         |
| [13]             | BC        | 20 (33.6)                          | 3                      | 0                      | 2         |
| [14]             | BC        | 10 (38.5)                          | 10                     | 10                     | 4.5       |
| [15]             | BC, UTUC  | 28 (0.03)                          | 10                     | 10                     | 3.3       |
| Present study    | BC, UTUC  | 10 (100)                           | 6                      | 5                      | 303.5     |

CTC = circulating tumor cell; UC = urothelial cancer; UTUC = upper tract urothelial cancer; BC = bladder cancer.
However, only 2 plasma-based cfDNA NGS studies have been reported for BC. Vanderkolkho et al. revealed an aggressive mutational profile in 51 metastatic BC with 95% of patients harboring deleterious alterations to TP53, RB1, or MDM2, and 70% harboring a mutation or disrupting rearrangement affecting chromatin modifiers such as ARID1A as well as targetable alterations in MAPK/ERK or PI3K/AKT/mTOR pathways, including amplification of ERBB2 (20%) and activated hotspot mutation in PIK3CA (20%) with the level of ctDNA in localized BC being significantly less to none compared to that in metastatic disease.[21] Another small study investigated genomic alterations in paired tissue and ctDNA from the plasma in 22 patients with metastatic BC and found frequent alterations in TP53 (50%), the TERT promoter (36.3%), the AT-rich interaction domain 1A (ARID1) (29.5%), FGFR2/3 (20.5%), PIK3CA (20.5%), and ERBB2 (18.2%) with significant discordance in the landscape between the two tests, while the tumor mutational burden was significantly associated with the number of genomic alterations in both tests.[21] Agarwal et al. detected an average of 5.4 genomic alterations per patient in 294 BC patients with most frequent alterations in TP53 (48%), ARID1A (17%), PIK3CA (14%), neurofibromin 1 (NF1) (12%), ERBB2 (10%), telomerase transcriptase (TERT) (10%), FGFR2 (10%), and FGFR3 (10%), which were similar to the above mentioned two historical tissue studies.[20,21] The prognostic significance of ctDNA alteration was proposed in 124 advanced UC patients. At least one genomic alteration was detected in 112 patients (90.3%). Commonly altered genes included TP53 (54.8%), PIK3CA (24.2%), ARID1A (22.6%), RB1 (19.9%), EGFR (16.1%), NF1 (13.7%), NDRG1 (12.9%), FGFR3 (11.3%), BRAF (10.5%), BRCAl (10.5%), and RAF1 (8.9%), the last two of which appear to be negatively associated with clinical outcomes.[21]

The DNA in the urine of patients with BC has also been studied, showing that cfDNA of the urine supernatant is more sensitive to tumor specific aberrations than cellular DNA of the urine precipitate because of reduced contamination from germline DNA of normal cells.[16] On the other hand, it was reported that the detection rate of the urine supernatant and precipitate by genomic profile is almost the same, although somatic mutations detected in both urine fractions are not necessarily the same probably due to contamination of normal alleles in the urine precipitate and different susceptibility of tumor cells to collapse (e.g., apoptosis and necrosis) thereby shedding tumor-derived DNA into the urine.[27] Togneri et al. demonstrated that clinically important genomic aberrations found in original tumor samples are mirrored in urinary DNA, and that the tumor genome is enriched in cfDNA compared with cellular DNA.[28] In line with these reports, we confirmed that more variant DNAs were found in the supernatant than in the precipitate. As long as the urine supernatant is not less sensitive in detecting tumor specific genetic alterations compared with the urine precipitate, urinary cfDNA may represent a reliable resource with less contamination of normal alleles for non-invasive genomic profiling in patients with UC.

We identified several uncommon genomic alterations for UC in the plasma or urinary cfDNA of our patients, APC, CSFR1, EGFR, FLT3, IDH1, JAK3, KDR, MLH1, NOTCH1, PDGFRα, RET, SMAD4, SMO, and STK11. Hotspot mutations were identified for IDH1, MLH1, and STK11, suggesting that these mutations may be directly actionable cancer-related genes. Although we did not confirm their gene expression in tumor tissue, the mutations isolated in the DNA may at least affect the amino acid or protein structure, which are likely to be possible targets for cancer therapy. The IDH1 mutation has not been previously reported in UC, but the IDH2 mutation was reported in adenocarcinoma of the bladder.[22] Mutations in IDH1/2 in the substrate binding site induce abnormal accumulation of D2-hydroxylglutatate, which is a competitive inhibitor of a-ketoglutarate dependent dioxygenases and in turn results in upregulation of proto-oncogenes such as HIF-1α, histone modification, and chromatin remodeling. IDH hotspot mutations are potential therapeutic targets for patients with glioma and acute myeloblastic leukemia.[19,20] Mutations in MLH1 together with MSH2, MSH6, or PMS2 are representative of the Lynch syndrome, which is an autosomal dominant cancer predisposition syndrome caused by germline mutations in the mismatch repair (MMR) genes and has increased risk of developing various cancers including in the urinary tract.[22] Recent studies advocated universal screening by immunohistochemistry followed by microsatellite instability testing for early detection of the Lynch syndrome since MMR protein loss was identified in 5%–9% of all UTUC patients.[23] Mutations in STK11 have been little investigated in UC. The STK11 gene encodes a serine/threonine protein kinase that regulates cell polarity and functions as a tumor suppressor. STK11 mutations are critical in lung cancer differentiation, tumorigenesis, and metastasis, and are a potential prognostic and predictive marker in non-small cell lung cancer.[22] A recent case report presented an unusual case of occult UC initially manifested as a multiorgan metastatic cancer without a detectable primary lesion in the urinary tract, in which targeted therapy with everolimus was given based on the mutation in STK11 detected by NGS.[25] Last but not least, it is relevant to discuss the differences in genomic alterations detected in CTC and ctDNA in the same blood samples. The majority of cfDNA (over 80% in healthy individuals) arises from hematopoietic cells.[24,28] Thus, differentiating a cancer-signal from normal background biological variation within an individual is a challenging issue to obtain the specificity of ctDNA detection. Normal hematopoietic cells accumulate somatic mutations during ageing which can lead to clonal expansions without dysplastic transformation, referred to as clonal hematopoietic mutations of indeterminate potential (CHIP).[29] CHIP presents a biological confounding factor for early cancer detection assays predicated on the characterization of cfDNA as tumor DNA based on somatic variant detection.[30] On this point, CTC may be a better resource for harvesting DNA since CTC contains complete genetic information, including the genome, transcriptome, and epigenome, which provides a more comprehensive genetic profile reflective of original tumors. However, CTC is extremely rare in the blood with 1–100 cells/mL among millions of WBC and billions of red blood cells, yielding a far less amount of DNA in pg order compared with that from cfDNA in ng order.[41] Nevertheless, our 6 patients with UC exhibited high CTC counts, which enabled us to compare mutation profiles of cfDNA and CTC isolated from the same blood sample. In all 6 patients, mutations detected in CTC were encompassed by cfDNA mutation profiles, and in 2 patients, cfDNA had extra mutations (in TP53 and FGFR3) other than those found in CTC. These mutations may have been simply missed by sampling error of the small number of CTC or allelic dropout during genome amplification may have prevented detection of somatic mutant alleles in CTC. Similar to our data, direct comparison of mutation profiles between CTC and cfDNA from the same blood sample from 6 metastatic breast
cancer patients with high CTC counts indicated that cfDNA sequencing finds more mutations than CTC and provides accurate reflection of mutations seen in CTC. Likewise, combination analysis of genomic profiles obtained from CTC and cfDNA in the same blood from 28 patients with head and neck or gastrointestinal cancer showed that the overall genetic mutational concordance between the two profiles was not so high (71.3%). CTC exhibited mutations that were not detected in cfDNA and vice versa. Taken together, genetic alteration profiles are unlikely to accurately correlate between CTC and cfDNA. Therefore, a combination assay could enhance the sensitivity of detecting genetic alterations, thus contributing to the development of precision medicine in cancer therapy.

There are several potential weaknesses to be acknowledged. Patient numbers were so small that a firm conclusion could not be made. We did not perform tissue confirmation of mutations detected in the plasma and urine cfDNA, although a significant discordance in the different NGS platforms with paired tissue and cfDNA has been reported using UC samples and genetic alteration in cfDNA is assumed to arise later and is absent in primary tumors. We did not address the prognostic significance of the identified mutations because of an insufficient follow-up period and limited number of patients with significant variability of treatments.

5. Conclusions
We demonstrated that the LiquidBiopsy Platform more efficiently detected CTC compared with previous studies using the CellSearch™ system and that NGS analysis using the Ion S5 system with Ion AmpliSeq Cancer Hotspot Panel allowed for detection of common and also rare clinically actionable mutations in CTC, plasma, and urine samples from patients with advanced UC. These findings support the pursuit of therapeutic strategies targeting these alterations to treat this highly malignant disease that is often refractory to conventional, non-targeted therapies.

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Statement of ethics
This study was approved by the Institutional Review Board of Dokkyo Medical University (# R-6-5). Informed consent was obtained from all subjects. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. None.

Conflict of interest statement
The authors have no conflicts of interest to declare.

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All authors contributed equally in this study.

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