Review Article on Urinary Biomarkers of Urological Malignancies

Toward urinary cell-free DNA-based treatment of urothelial carcinoma: a narrative review

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Abstract: Liquid biopsy technique targeting urinary cell-free DNA (cfDNA) is getting a lot of attention to overcome limitations of the present treatment strategy for urothelial carcinoma, including urothelial bladder carcinoma (UBC) and upper tract urothelial carcinoma (UTUC). Analysis of tumor-derived DNA in urine focusing either on genomic or epigenomic alterations, holds great potential as a noninvasive method for the detection of urothelial carcinoma with high accuracy. It is also predictive of prognosis and response to drugs, and reveals the underlying characteristics of different stages of urothelial carcinoma. Although cfDNA methylation analyses based on a combination of several methylation profiles have demonstrated high sensitivity for UBC diagnosis, there have been few reports involving epigenomic studies of urinary cfDNA. In mutational analyses, frequent gene mutations (*TERT* promoter, *TP53*, *FGFR3*, *PIK3CA*, *RAS*, etc.) have been detected in urine supernatant by using remarkable technological innovations such as next-generation sequencing and droplet digital PCR. These methods allow highly sensitive detection of rare mutation alleles while minimizing artifacts. In this review, we summarize the current insights into the clinical applications of urinary cfDNA from patients with urothelial carcinoma. Although it is necessary to conduct prospective multi-institutional clinical trials, noninvasive urine biopsy is expected to play an important role in the realization of precision medicine in patients with urothelial carcinoma in the near future.

Keywords: Cell-free DNA (cfDNA); liquid biopsy; urothelial carcinoma; urine; *TERT* promoter; *TP53*; *FGFR3*; methylation

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Introduction

Approximately 95% of urothelial carcinoma (UC) occurs in the lower urinary tract [urothelial bladder carcinoma (UBC)], whereas those in the upper urinary tract [upper tract urothelial carcinoma (UTUC)] are relatively rare (1).

UBC can be classified into two major groups: non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). Because NMIBC is associated with fewer deaths and a high risk of intravesical recurrence even after transurethral surgery, ranging from approximately 50% to 80% (2,3), patients with NMIBC require continuous follow-up with cystoscopy and urine cytology, making NMIBC one of the most prevalent and the

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most expensive cancer all over the world (4). For patients with localized MIBC, radical cystectomy, lymph node dissection, and cisplatin-based neoadjuvant chemotherapy (NAC) are the gold standard management proposed by current clinical guidelines (5-11). On the contrary, radical nephroureterectomy (RNU) with an ipsilateral bladder cuff and retroperitoneal lymph node dissection is one of the standard therapies for patients with muscle invasive, high-grade, or bulky UTUC, whereas kidney-sparing surgery (KSS) plays an increasingly important role in the management of low-risk UTUC, mainly due to improvements in endoscopic technologies (12). Even though the stage and grade of UTUC are decided by radiographical imaging or pathological tests obtained by endoscopic biopsy specimens, it is often difficult to determine accurately due to the thin wall of the upper urinary tract and the low volume of biopsy specimens (13). Because of the risk of recurrence during the follow-up period after RNU, surveillance based on cystoscopy, radiographical tests, and urinary cytology is mandatory. When KSS is performed, careful surveillance of the ipsilateral upper urinary tract is also necessary due to the high risk of local disease recurrence (12).

For these reasons, patients with UC, including UBC and UTUC, need to undergo several procedures at the time of initial diagnosis and continuous disease surveillance followed by initial therapy. Despite several commercially available assays for the detection of UC, such as UroVysion (14-20), urine cytology remains the most accessible and widely used noninvasive test worldwide. For the detection of UC, urine cytology established by The Paris System has high sensitivity for high-grade tumors (84%), but low sensitivity for low-grade tumors (16%) (21,22). Since current procedures for diagnosis or surveillance, such as cystoscopy or ureteroscopy (for patients after KSS) are invasive and costly, less invasive, and reliable follow-up methods are necessary for patients with (especially for low-grade) UC. Messer *et al.* reported that urine cytology is less sensitive in detecting UTUC than UBC and should be performed in selected cases of UTUC (23). In addition, there are no reliable prognostic biomarkers that reflect tumor grade or stage for patients who might need neoadjuvant or adjuvant chemotherapy. For these reasons, there is an urgent need to develop a noninvasive biomarker that could accurately diagnose, predict prognosis and response to drugs, and reveal the characteristics of the various stages of UC.

Liquid biopsy is a novel technology that detects and analyzes small amounts of tumor-derived cell-free DNA (cfDNA), extracellular vesicles (EVs), and circulating tumor cells (CTCs) in body fluids such as blood and urine (24). Recent technological innovations, such as the development of next-generation sequencing (NGS) or droplet digital PCR (ddPCR), have made it possible to perform molecular analysis of trace amounts of cell-free DNA in body fluids with high sensitivity. Liquid biopsy is expected to be indispensable for the realization of precision medicine (25,26). Owing to the fact that UC, unlike other carcinomas, is constantly in contact with urine, urinary cfDNA is an important source of genomic analysis of UC (27) (*Figure 1*). In this review, we summarize the current status, potential clinical applications, and limitations of cfDNA in urine as biomarkers in patients with UC. We present the following article in accordance with the Narrative Review reporting checklist (available at http://dx.doi.org/10.21037/tau-20-1259).

Literature search methodology

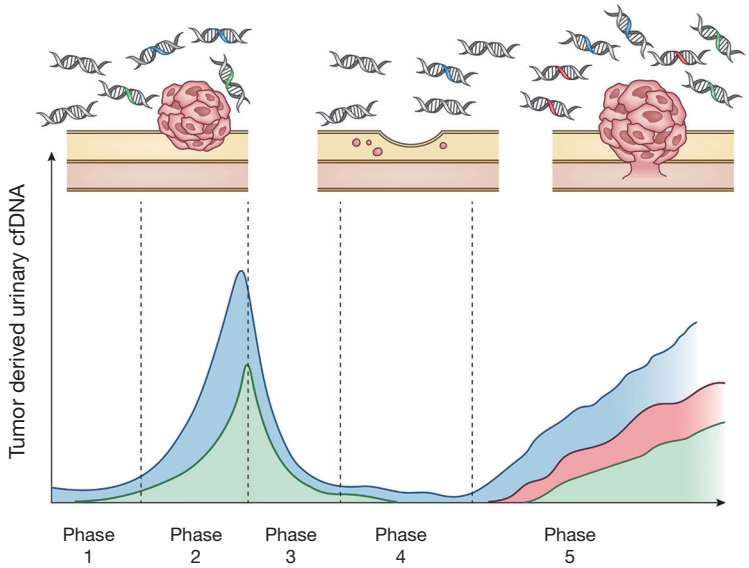
We conducted a literature review of cfDNA about urothelial carcinoma. The literature was searched for publications up to May, 2020 for all English-language journals relating to cfDNA, using the PubMed database. The specific terms used were “bladder cancer” OR “bladder carcinoma” OR “urothelial cancer” OR “urothelial carcinoma” OR “upper tract” combined with “free DNA” OR “circulating DNA” OR “plasma DNA” OR “serum DNA” OR “urine DNA” OR “urinary DNA” OR “liquid biopsy” OR “liquid biopsies”.

Molecular biology of urothelial carcinoma tissue

Due to technological advancements, NGS has made it possible to offer a comprehensive molecular characterization of various tumors, including UC. Over the last decade, molecular analysis of tumor specimens of UC has revealed details of genetic and epigenetic profiles and the association between molecular profiles and environmental factors. The classification of UC into molecular-based subtypes has facilitated more precisely decided treatment for patients with UC in addition to the prediction of prognosis, therapy resistance, and tumor progression (28-35).

Genetic analysis

Using genome and transcriptome profiling, Robertson *et al.* demonstrated that the overall mutational burden in bladder cancer is associated with APOBEC-signature



Phase 1	Earlier detection
Phase 2	Molecular profiling, Prognostication
Phase 3	Residual tumor detection, Precision adjuvant intravesical therapy
Phase 4	Surveilance, Prognostication
Phase 5	Molecular profiling, Precision peri-surgical systemic therapy

Figure 1 Applications of urinary cfDNA analysis during UC management.

mutagenesis, and certain mutation signatures with a high-mutation subset are associated with better survival outcome in patients with MIBC (30). Hedegaard *et al.* also reported that the identification of molecular subtypes could offer better treatment strategies and better prognostication for patients with NMIBC (33). Hassler *et al.* compared the molecular characteristics of UBC and UTUC. Although UTUC and UBC share several similar genomic alterations, some molecular profiles are unique to UTUC-associated syndromes (34). Lynch syndrome-associated UTUC has germline mutations in mismatch repair genes or microsatellite instability. Aristolochic acid-induced UTUC has frequent A>T conversion or rare FGFR3 mutations. NGS analysis revealed several representatives of somatic mutations in UC. Mutations in the *TERT* promoter region are one of the most frequently occurring mutations in various neoplasms, including UC (36). Hotspot mutations in the *TERT* promoter mainly affect two regions, chromosome 5 of g.1295228 C>T and g.1295250 C>T, 124 and 146 upstream of bases of transcription start site (36,37). The mutant *TERT* promoter allele alters the binding capacity of transcription factors and engages in long-range chromatin interactions, subsequently stimulating *TERT* promoter activity and enabling tumors to overcome the end-replication problem and avoid senescence (38-40). *TERT* promoter mutations are widely identified in low-grade tumors, high-grade tumors, or rare histological variants (41-46). For these reasons, *TERT* promoter mutation is thought to be an early event in UC carcinogenesis (47).

PIK3CA is activated by point mutations at higher frequencies in NMIBC than in MIBC. *PIK3CA* mutations are most commonly associated with *FGFR3* or *RAS* mutations. *PIK3CA* has domains for binding to RAS. There may be cooperation between *PIK3CA* mutant proteins and other events that are known to activate RAS (48).

RAS gene mutations (*KRAS* or *HRAS* mutations) are also frequently found in UBC. The Ras superfamily of monomeric G proteins has been reported to contribute to UBC progression with epidermal growth factor receptor, p53, and PTEN (49). *TP53* mutations are rarely detected in low-grade UC, but are frequently detected in high-grade UC, including muscle invasive disease (30,33). *TP53* mutations are also detected in carcinoma in situ (CIS) and are considered to be early events of CIS tumorigenesis. The primary function of *TP53* genes is to control cell cycle progression, senescence, and cell death, leading to inhibition of tumorigenesis (50). These genes are activated by various cellular stresses, including DNA damage, hypoxia, replicative stress, and oncogene expression. A common cause of TP53 loss-of-function is an inactivating missense mutation. Fibroblast growth factor receptor 3 (FGFR3) is a glycoprotein composed of three extracellular immunoglobulin-like domains and a split tyrosine-kinase domain. An activating hotspot mutation in the *FGFR3* gene or chromosomal translocation with a breakpoint near *FGFR3* is frequently identified in UC (30,51). *FGFR3* is

characterized by a high prevalence of low stage tumors; these mutations are more frequently identified in pTa (80%) than in MIBC (10–20%). Given these facts, urinary cfDNA would help to differentiate low-grade tumors from high-grade tumors by analyzing *TP53* and *FGFR3* mutations.

Epigenetic analysis

In a previous study, Wolff *et al.* observed that 12% of all CpG loci were hypermethylated in bladders with cancer (52). They also reported that carcinogen exposure rather than a clonal expansion seems to result in these epigenetic alterations. DNA methylation is the most studied epigenetic modification in cancer and is thought to be a prevalent and early event in tumorigenesis (53). Hypermethylation of CpG islands represses tumor suppressor gene transcription either directly by inhibiting transcription factor binding or indirectly through the recruitment of methyl-CpG-binding proteins. Hypomethylated CpG regions might activate oncogenes, resulting in chromosome instability (54,55). Although methylation-specific PCR (MSP) is a sensitive method for detecting a methylated region of interest using specific primers on bisulfite-converted DNA, recent technological advancements in bisulfite sequencing have made it possible to analyze methylation of DNA on a genome-wide scale (56). These genome-wide methylation analyses will lead to a deeper understanding of how the genetic function is implemented and regulated. Epigenetic alterations in the promoter regions of susceptible genes can be detected in both blood and urinary cfDNA, including key cellular pathways such as cell cycle control, apoptosis, cell differentiation, DNA repair, cellular adhesion, and migration.

Overview of the cell-free DNA

The cfDNA released into body fluids by apoptosis, necrosis, and secretion of both cancerous and noncancerous tissues is free from any type of cells as fragmented nucleic acids (24). The presence of tumor-derived cfDNA in serum was first reported by Leon *et al.* in 1977 (57). Due to the minimal amount of cfDNA in body fluids and the requirement of a sensitive assay for analysis, the research in this field was limited for a while. In 1997, Lo *et al.* reported for the first time that fetal cfDNA circulates in maternal plasma (58), which led to the development of noninvasive prenatal genetic testing. Advances in obstetrics and genetic analysis techniques have led to rapid progress in research on tumor-derived cfDNA.

The analysis of cfDNA has several advantages over conventional tissue biopsy. It allows us to analyze in a less invasive, rapid, repeated, sequential manner, and also to overcome the heterogeneity of tumors (59). Thus, cfDNA can provide useful genetic information and offer several advantages not only as a noninvasive diagnostic tool, but also as an early indicator of minimal residual disease, recurrence, drug resistance, or metastasis, and believed to replace tissue biopsy in the near future.

Although cfDNA can be detected in both plasma and serum, plasma samples are preferred over serum for cfDNA analysis because blood cell lysis during the preparation of serum samples could release DNA from noncancerous cells, which might lower the sensitivity of cfDNA detection derived from tumors (60).

However, limited DNA quantity in plasma makes it difficult to detect mutations with very low variant allele frequency or those of localized tumors because the amount of cfDNA in plasma is mainly correlated with tumor burden in various types of malignancies. For these reasons, the majority of previous studies focused on advanced tumor stages, and the clinical utility of cfDNA mutational analysis in blood for the detection of early-stage tumors is less well documented (61). Still, owing to the fact that UC, unlike other carcinomas, is in constant contact with urine, urinary cfDNA is an important source of genomic analysis of UC (27).

DNA sources in urine consist of both urinary sedimented cells (pellet DNA) and urinary supernatant (cfDNA) separated by centrifugation, and the utility of pellet DNA analysis has long been established (62,63). Importantly, urinary cfDNA in the supernatant, discovered later than the pellet, has been reported to exhibit a higher sensitivity than the pellet DNA in several papers (64-66). Urinary cfDNA can be classified into two groups according to their fragment size: high-molecular-weight (1 kbp or longer) and low-molecular-weight (67,68). High-molecular-weight urinary cfDNA is derived from necrotic cells or lymphocytes in the urinary tract (67,69). However, low-molecular-weight urinary cfDNA can originate either from apoptotic cells or systemic circulation (67,70). Bryzgunova *et al.* estimated that more than 3×10^6 epithelial cells could be excreted into the urine in one day, and some of these epithelial cells undergo apoptosis to release cfDNA into the urine (71). Furthermore, urinary cfDNA carries genetic information of circulating cfDNA that passes through the glomerular barrier, known as trans-renal DNA (69). For example, *EGFR* mutations have been detected in the urinary cfDNA of patients with non-small-cell lung cancer (71),

and *KRAS* mutations have been detected in patients with colorectal cancer (66,72) and pancreatic cancer (73). More importantly, urine has the advantage of being a noninvasive sample source over tissue and blood. Urine can be collected in cups without any specialized skills or equipment.

Techniques for efficient detection of urinary cfDNA

In order to detect urinary cfDNA efficiently, some techniques are essential for urine storage, isolation of urinary cfDNA, and detection method. Since voided urine contains a lot of contaminants like bacteria, blood cells, or crystals, it is necessary to exclude artifacts derived from these contaminants as much as possible. Because voided urine samples from patients are susceptible to crystal precipitation even after refrigeration at 2 to 8 ℃ for several hours, urine sample processing, including centrifugation and removal of crystals and cellular components followed by storage at −80 ℃, should be performed promptly after urine collection (74,75). Although cfDNA can be isolated by classical laboratory techniques, many types of commercial kits are available for the isolation of cfDNA. For the detection of genomic alterations efficiently, NGS and ddPCR technology are widely used. Each of these methods has its own advantages and features that need to be well understood. NGS is a high-powered analysis method capable of running millions of sequences simultaneously, enabling comprehensive detection of gene mutations. Furthermore, ddPCR allows for highly accurate measurements of DNA content by dividing a nucleic acid sample into thousands of parallel PCR reactions, each of which is read separately and analyzed statistically. Since the ddPCR technique is based on a given specific primer, it is not suitable for extensive exploratory measurements; however, it offers higher accuracy and sensitivity than other methods.

Association of genomic alteration profiles between tumor tissue and cfDNA

Several researchers have investigated the concordance of genomic alteration profiles between tumor tissue and cfDNA. A previous study by Agarwal *et al.* analyzed the comprehensive genomic profile of plasma cfDNA in metastatic UC, including UTUC, and compared the frequency of genomic mutations between tumor tissue and cfDNA (76). They observed a similar frequency of recurrent mutations (*TP53*, *ARID1A*, *PIK3CA*, *ERBB2*, and *FGFR3*) between tumor tissues of patients with MIBC and cfDNA of patients with metastatic UBC. Dudley *et al.* observed a certain concordance of mutations in tumor tissue and urinary cfDNA obtained from paired urine and tissue samples of 18 patients with UBC. They also reported that tumor mutations also identified in urine had a higher median allele fraction (27% *vs.* 9%, P<0.0001) than those not identified in urine, indicating that genomic concordance between mutations found in tumor tissue and urinary cfDNA is higher for truncal mutations (77). Cheng *et al.* investigated the genome-wide analysis of methylation status of urinary cfDNA in patients with UBC. Shallow-depth paired-end genome-wide bisulfite sequencing revealed that the tumor tissue and urinary cfDNA have a significant positive correlation in methylomes (78).

In short, there seems to be an overall concordance between tumor tissue and cfDNA in terms of genomic alterations, including genomic mutations and methylation alterations.

Clinical application of cfDNA analysis for patients with UBC

Several methods for cfDNA analysis have been reported. In the pre-NGS era, when devices such as NGS or ddPCR, which can detect a small number of mutated alleles, were not prevalent, microsatellite, methylation, or quantification of blood cfDNA were mainly investigated for clinical use.

Quantification of cfDNA

Several studies have demonstrated the quantification of cfDNA. Brisuda *et al.* reported that urinary cfDNA levels in patients with UBC were significantly higher than those in the control group, with a sensitivity of 42.4% and a specificity of 91.2%. However, the levels of urinary cfDNA are affected by hematuria or pyuria, and attention is necessary for clinical use (79).

Microsatellite analysis

Microsatellite analysis was performed in the early era of cfDNA investigation. Microsatellites are highly polymorphic DNA-repeat regions, and alterations of microsatellite DNA are thought to be valuable markers for detecting UC (80,81). Utting *et al.* reported that multiple

Table 1 Urinary cfDNA methylation panels and clinical utilities for urothelial carcinoma

Cancer type	Cohort	Sample	Method	Target	Sensitivity	Specificity	Reference
UBC	46 UBC, 39 hematuria	Urinary cfDNA	GWBS	GWBS	93.5%	95.8%	(78)
UBC	263 urine samples	Urinary cfDNA	PCR, MSP	*FGFR3* mutation *SEPTIN9*, *HS3ST2* and *SLIT2*	–	–	(84)
UBC	14 UBC, 12 hematuria	Urine pellet, urinary cfDNA	MSP	GHSR/MAL	AUC: 0.83	–	(85)

GWBS, genome wide bisulfite sequence.

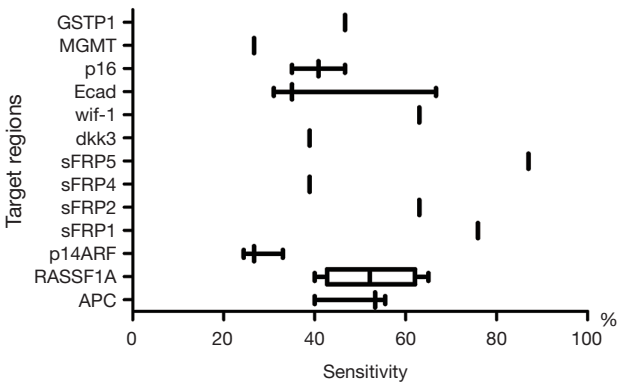


Figure 2 Reported sensitivities of methylation analysis of urine DNA.

simultaneous investigations of microsatellite markers of urinary and plasma cfDNA have clinical potential for noninvasive diagnosis of UBC (82). Szarvas *et al.* reported that microsatellite analysis of urinary cfDNA is more sensitive for the detection of UBC than that of urine pellet DNA (66).

Integrity of cfDNA

It has been reported that cfDNA from apoptotic cells is highly fragmented, whereas cfDNA from necrotic cells results in longer DNA fragments. Casadio *et al.* reported that the DNA integrity of urinary cfDNA showed a sensitivity of 73% and a specificity of 83% in symptomatic patients. In their report, urinary cfDNA integrity was defined as the quantity of *c-Myc*, *BCAS1*, and *HER2* with sequences longer than 250 bp by real time PCR (83).

Epigenetic alterations in cfDNA

To date, there have been few reports on the epigenetic analysis of urinary "cfDNA" (*Table 1*) (78,84,85), but many reports on urinary "pellet" DNA (86-101) using various ranges of methylation biomarkers. The combination of multiple methylation levels showed a high sensitivity for UBC diagnosis. However, the sensitivity and specificity of each single methylation marker differ across several studies (*Figure 2*). Commercially available AssureMDx (MDx Health, USA) evaluated the DNA methylation of *OTX1*, *ONECUT2*, and *TWIST* and mutation analysis of *FGFR3*, *TERT* promoter, and *HRAS* in urinary pellet DNA for the detection of UC (101). The sensitivity and specificity of AssureMDx were 97% and 83% in 74 patients with UBC and 80 patients with gross hematuria, respectively. Because hematuria is an important symptom of UC, this commercial assay has great potential for clinical use, reducing unnecessary cystoscopies in patients with hematuria. In comparison with studies on epigenomics in urinary pellet DNA, few reports have been made about urinary cfDNA. Cheng *et al.* reported high sensitivity and specificity of shallow-depth genome-wide bisulfite sequencing of urinary cfDNA (78). In serum cfDNA, hypermethylation of 3 genes (*APC*, *GSTP1*, and *TIG1*) provided not only diagnostic but also valuable prognostic information for patients with UBC (102). Others reported that the methylation levels of *p16* and *DAPK* can be used to differentiate NMIBC from healthy controls with 76.2% sensitivity and 100% specificity (103). Furthermore, the point that *DAPK* promoter methylation was detected in the serum of 71.4% of patients with low-grade NMIBC is worth noting. Thus, a unique feature of the methylation assay is that it can be performed on blood samples, even in patients with early stages of UC. However, there are certain issues with methylation analysis that need to be solved. First, there is great variability in the positive rate in each study. Second, unlike genetic mutations, the changes are not concentrated in a specific locus, and last, considerable DNA losses occur under bisulfite treatment.

Table 2 Urinary cfDNA mutational panels and clinical utilities for urothelial carcinoma

Cancer type	Cohort	Sample	Method	Sensitivity	Specificity	Reference	Note
MIBC	17	p, ucf	WGS	–	–	108	–
Mostly NMIBC	143 UC, 144 control	p, up, ucf	Targeted sequence (*TERT* promoter)	81.8%	97.7%	107	**
NMIBC	25	p, ucf	Targeted sequence (71 genes)	–	–	109	–
NMIBC (cystectomy)	363 NMIBC, 468 cystectomies	p, ucf	Personalized ddPCR	NA	NA	106	–
UBC	23	up, ucf	Oncoscan	90%	–	64	***
UBC	150 UBC, 52 hematuria	ucf	ddPCR (*TERT* promoter, and *FGFR3*)	85.9%*	100%	105	–
UBC	6 progress, 6 recurrence	p, ucf	Personalized ddPCR	NA	NA	110	–
UBC	9 cystectomies	ucf	Targeted sequence (9 genes)	–	–	111	–
UBC	104	ucf	ddPCR (*TERT* promoter)	68.3%	–	112	–
UBC	53 UBC, 36 control	up, ucf	Targeted sequence (*TERT* promoter)	63%	–	115	****
UBC	118 UBC, 67 control	ucf	Targeted sequence (460 genes)	93%	96%	77	–
UBC	92 BT, 33 control	up, ucf	Targeted sequence (5 genes)	–	–	116	–
UC	65 UC, 198 control	up, ucf	WGS	86.5%	94.7%	65	–
UTUC	56 UTUC, 50 hematuria, 21 surveillance, 26 control	ucf	ddPCR (*TERT* promoter, *FGFR3*, and *PIK3CA*)	78.6%*	96%	113	–
UTUC	26	ucf	WGS	–	–	114	–

*, combined with urine cytology; **, the sensitivity was 66–83.5% in urine pellet, and 7.1% in p.; the specificity was 94.6–100% in urine pellet, and 98.7% in p.; ***, the sensitivity was 61% in urine pellet; ****, the sensitivity was 77% in urine pellet. p, plasma; up, urine pellet DNA; ucf, urinary cfDNA; WGS, whole genome sequence.

Genetic alterations in the cfDNA

In order to detect low volumes of mutant DNA in urine, targeted panel methods are often used to ensure the accuracy and efficiency of genetic analysis of DNA in urine (77,104). There have been several reports about the clinical utility of urinary cfDNA in patients with UC (64,65,77, 105-116) (*Table 2*). Because genetic alterations are highly specific to cancer, it is possible to increase the sensitivity of UBC detection by examining many mutations while maintaining specificity (77,105). For this reason, it is necessary to balance the cost by determining the number of genetic mutations to be analyzed. Due to the limited number of frequent genetic alterations in UBC, more genetic alteration data on urinary cfDNA have been accumulated from various institutions at various stages of cancer than that of epigenome analysis (*Figure 3*).

Dudley *et al.* used high-throughput targeted sequence CAPP-Seq assay to detect frequent mutations in urinary cfDNA for diagnosis and surveillance of UBC (77). The sensitivity of CAPP-Seq was 84–93%, and the specificity was 96–100%. They also reported that this targeted sequence could detect 91% of UBC recurrence in the surveillance setting, and significantly outperformed current clinical tests such as UroVysion or urine cytology. Christensen *et al.* reported the clinical utility of personalized cfDNA analysis for disease surveillance of patients with UBC (106). They developed urine and plasma cfDNA hotspot mutational assays by ddPCR for patients with *FGFR3* and *PIK3CA* tumor mutations, and reported that these mutations in urinary cfDNA were significantly associated with disease progression from NMIBC. Furthermore, a positive correlation of tumor-derived DNA between urine and plasma was observed. We have reported the utility of ddPCR-based simple urinary cfDNA assay targeting 3 mutation sites (2 promoter mutations of *TERT* and 1 *FGFR3* mutation) (105). In this report, the sensitivity was 77.5% to 85.9% in conjunction with urine cytology, and the specificity was 100%. Furthermore, in disease surveillance after transurethral resection of bladder tumor, patients with mutant *TERT* C228T positive urinary cfDNA

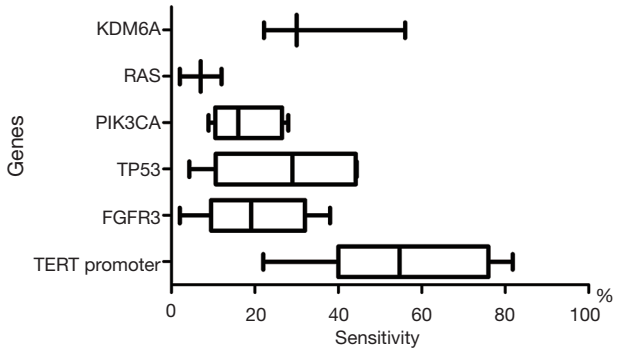


Figure 3 Reported sensitivities of mutational analysis of urinary cfDNA.

had a significantly worse prognosis for bladder tumor recurrence than patients with negative mutations. Since *TERT* promoter mutations are frequently detected in UC, *TERT* promoter mutation is thought to be one of the strong candidates for noninvasive biomarkers in urinary cfDNA in many reports (105,107,112,115).

Clinical application of cfDNA analysis in UTUC

Due to the small number of patients with UTUC, there have been few reports on urinary cfDNA analysis in UTUC. We investigated the diagnostic and prognostic potential of urinary cfDNA in patients with localized UTUC (113). In our report, the sensitivity of 3 hotspot gene mutations in urinary cfDNA was only 55.4%, but the sensitivity increased to 78.6% when combined with urine cytology. Interestingly, urinary cfDNA collected after radical nephroureterectomy was a predictor of bladder tumor recurrence after radical surgery. Lu *et al.* investigated the whole genome sequence of urinary cfDNA in Chinese patients with UTUC (114). They concluded that a mutational signature in urinary cfDNA induced by aristolochic acid might serve as a screening tool to define low-risk UTUC with therapeutic relevance. Furthermore, this mutational signature in urinary cfDNA is useful for diagnostic uncertainty when kidney-sparing treatment and/or systemic therapy are considered.

Challenges and future directions for urinary cfDNA analysis

Current diagnostic procedures have some issues to be solved. First, urine cytology has low sensitivity for low-grade tumor; second, urinary tract endoscopy is invasive; third, BCG-induced inflammatory changes in the urothelium are often mistaken for CIS or other malignant manifestations on cystoscopy. Urinary cfDNA analysis can compensate for the weaknesses of current procedures and strengthen diagnostic capabilities, as proposed by many guidelines. It is necessary to conduct prospective multi-institutional clinical trials that address specific clinical questions in an accurate and precise manner because current reports about urinary cfDNA are mainly based on a small number of patients in a single institute. Urine cytology has a high specificity and a long history of clinical use. We believe that in the future, urinary liquid biopsy can help in improving diagnosis by combining morphological evaluation of urine cytology and molecular assessment of urinary cfDNA. *TERT* promoter mutations have been detected in the non-malignant urothelium of patients with UBC, and these mutations are significantly associated with bladder tumor recurrence (117). The fact that urinary cfDNA alterations are detected in patients without visible tumor indicates that these mutated cfDNAs originate from non-malignant urothelium, reflecting the whole bladder urothelium genomic status. Though this might reduce positive predictive value, patients with mutated cfDNA in urine without visible tumor have a high likelihood of developing tumors and should be followed carefully. Furthermore, it might be useful for patients with MIBC after bladder preservation therapy by urinary cfDNA analysis. Urinary cfDNA analysis may offer not only a novel initial workup for patients suspected of having UC, but also a novel follow-up strategy or novel adjuvant therapy for patients in the surveillance.

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