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Circulating and urinary tumour DNA in urothelial carcinoma — upper tract, lower tract and metastatic disease

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Abstract

Precision medicine has transformed the way urothelial carcinoma is managed. However, current practices are limited by the availability of tissue samples for genomic profiling and the spatial and temporal molecular heterogeneity observed in many studies. Among rapidly advancing genomic sequencing technologies, non-invasive liquid biopsy has emerged as a promising diagnostic tool to reproduce tumour genomics, and has shown potential to be integrated in several aspects of clinical care. In urothelial carcinoma, liquid biopsies such as plasma circulating tumour DNA (ctDNA) and urinary tumour DNA (utDNA) have been investigated as a surrogates for tumour biopsies and might bridge many shortfalls currently faced by clinicians. Both ctDNA and utDNA seem really promising in urothelial carcinoma diagnosis, staging and prognosis, response to therapy monitoring, detection of minimal residual disease and surveillance. The use of liquid biopsies in patients with urothelial carcinoma could further advance precision medicine in this population, facilitating personalized patient monitoring through non-invasive assays.

Sections

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Key points

- Genomic profiling in urothelial carcinoma has enabled precision medicine to transform the management of this malignancy.

- Liquid biopsies are non-invasive genomic assays that serve as surrogates for the primary tumour biopsy. In urothelial carcinoma, liquid biopsies include urinary tumour DNA (utDNA) and circulating tumour DNA (ctDNA).

- Advances in genomic sequencing techniques have enabled researchers to detect utDNA and ctDNA at previously undetectable levels. Genomic profiling of the primary tumour facilitates the creation of bespoke utDNA and ctDNA panels for patients with urothelial carcinoma.

- In urothelial carcinoma, utDNA and ctDNA are very promising in multiple areas of care including diagnosis, risk stratification and prognostication, monitoring of response to systemic therapy, detection of minimal residual disease and surveillance.

- Results from different studies have shown that utDNA and ctDNA outperform conventional markers for diagnosis and surveillance, showing promise for the integration of these factors into management paradigms. For example, utDNA and ctDNA consistently outperform urine cytology, and the detection of these markers identifies recurrence seen through cross-sectional imaging, holding implications for early and personalized systemic therapies.

Introduction

New understanding of molecular carcinogenesis and therapeutic resistance mechanisms as well as advances in genomic sequencing technologies have introduced a new era of precision medicine. Several tissue-based biomarkers have been established for diagnostic, prognostic or predictive purposes in the management of various tumours, including urothelial carcinoma[1]. As clinicians become accustomed to using these biomarkers for cancer management, familiarity with the various assays available and access to these instruments have become points of discussion. Urologists have traditionally exploited the natural orifices of the urinary tract to atraumatically access urothelial malignancies originating from both the upper and lower urinary tracts. Nevertheless, collecting tissue and tumour quantity adequate for genomic profiling requires repeated tissue sampling, which poses considerable logistical, risk and cost barriers. Thus, cell-free DNA (cfDNA), along with the rapid technological advances that have enabled increasing sensitivity and specificity in cfDNA sequencing, has attracted great interest in both the research and clinical communities[2].

Owing to the novelty of liquid biopsies and the developing literature, the nomenclature associated with these genomic tests remains inconsistent. cfDNA refers to extracellular DNA detected from a source of tissue or fluid. In characterizing a malignancy, DNA derived from tumour in the blood is denoted as circulating tumour DNA (ctDNA) and in the urine is denoted as urinary tumour DNA (utDNA). In the circulating blood, a majority of cfDNA is derived from native lymphocytes, and differentiating native-derived components is crucial for distinguishing tumour from native DNA during malignancy detection[3–5]. In the blood, ctDNA might be detected either in plasma or serum, although plasma is most commonly analysed in current studies[6,7].

Numerous questions regarding the biology of cfDNA in the context of urothelial carcinoma as well as the clinical utility of cfDNA remain unanswered. Examples include the validity of cfDNA to represent the genomic profile of a primary tumour, the significance of detectability following definitive treatment of urothelial carcinoma and the role of cfDNA in surveillance protocols. Following the success of plasma ctDNA implementation in tumour genomic profiling in several cancers such as lung, colon and breast[8], the possibility of integrating ctDNA into the current clinical paradigm of urothelial carcinoma is being described in a growing body of literature. Specifically, urinary cell-free DNA (utDNA) can be repeatedly and easily sampled, and might serve as a valuable surrogate for tissue-based genomic interrogations.

In this Review, we provide an overview of the genomic landscape in urothelial carcinoma, the biological properties of cfDNA and the different liquid biopsy technologies available (Fig. 1). We review the current state of ctDNA and utDNA research and clinical applications in urothelial carcinoma of the upper and lower urinary tracts (Tables 1 and 2). Based on the insights derived from existing studies, we speculate on future perspectives of ctDNA in clinical trials and practice.

Genomic landscape of urothelial carcinoma

Advances in tumour sequencing enabled researchers to understand the genomics and biology of urothelial carcinoma. By identifying genomic profiles and differences in gene expression, clinicians might direct therapy to target gene mutations and expression. Within urothelial carcinoma, genomic landscape, mutational burden and gene expression are different among the upper tract, lower tract and metastatic setting, although these tumours share similar anatomical sources of origin.

Non-muscle-invasive bladder cancer

Non-muscle-invasive bladder cancer (NMIBC) constitutes ~75% of all new bladder cancer diagnoses[9]. This disease category encompasses a heterogeneous patient population with different tumour grades and stages[10]. Cancer recurrence is very common, and occurs in approximately half of all patients with NMIBC[11]. The most common somatic alterations in patients with NMIBC are found in the *TERT* promoter region (73–79%), and in genes including *FGFR3* (30–64%), *KDM6A* (38%), *PIK3CA* (24–26%), *STAG2* (23%), *ARID1A* (21%), *TP53* (21–26%) and *RAS* (11%)[12,13]. Interestingly, some mutations commonly observed in patients with NMIBC (for example, mutations in the *TERT* promoter and chromatin modifier genes) can also be detected in histologically normal urothelium, suggesting that these genomic alterations might precede malignant transformation[14–16]. Overall patterns of genomic alterations might be both prognostic and predictive of response to therapy. For instance, high-grade NMIBC has a higher tumour mutational burden (TMB) than low-grade NMIBC[13]. However, a reduced TMB has been observed in tumours refractory to intravesical immunotherapy, possibly owing to the limited neoantigen burden and immunogenicity of these tumours[17].

Different disease subtypes can also harbour distinct genomic alterations. Low-grade tumours are more likely to harbour mutations in *FGFR3* and chromatin remodelling genes (such as *KDM6A*)[12,13,18] than high-grade tumours, in which, conversely, *TP53*, *MDM2* and *ARID1A* alterations are particularly frequent[12,13]. The prevalence of alterations in cell-cycle regulator genes (*RB1*, *CCND1*, *p21* or *CDKN2A*) increases with increasing grade and stage, occurring in 13% of low-grade Ta disease, 41% of high-grade Ta disease, 42% of high-grade T1 disease and 53% of muscle-invasive bladder cancers (MIBCs)[13]. With regard to therapy response, patients with NMIBC harbouring *ARID1A* mutations

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in pretreatment index tumours were observed to be refractory to intravesical bacillus Calmette–Guérin (BCG) treatment (HR 3.14)[13], with substantially shorter time to relapse than patients with wild-type *ARID1A*[18]. In a study in which genomic profiling of recurrent NMIBC tumours following intravesical BCG treatment was performed, patients with alterations in *TP53, CCNE1, CDKN2A* and *CDKN2B*, and *APOBEC* showed higher propensity for progression to MIBC than patients without these alterations[17–19]. Overall, the identification of the most common somatic alterations in NMIBC tumours helps in the prognostication, risk stratification and prediction of response to therapy. Additionally, understanding the genomic landscape of NMIBC tumours can help distinguish the genomic alterations and TMB from those of MIBC and upper tract urothelial carcinoma (UTUC).
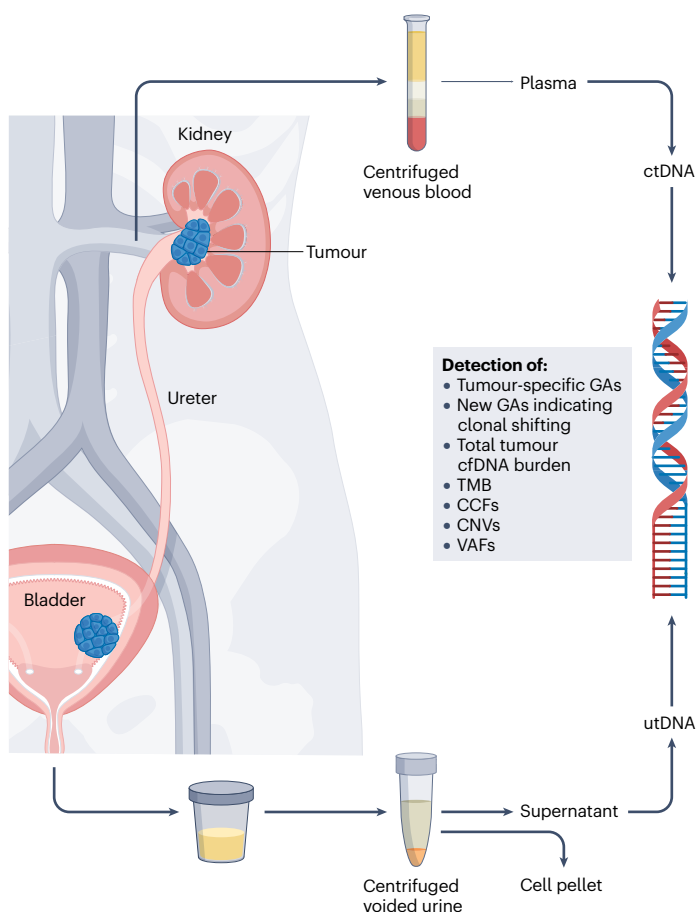

Fig. 1 | Cell-free DNA assays in urothelial carcinoma. Venous plasma can be used to detect circulating tumour DNA (ctDNA), whereas the urine supernatant from voided urine is used to detect urinary tumour DNA (utDNA). Both utDNA and ctDNA might undergo sequencing or candidate gene analysis through methods including urinary cancer personalized profiling by deep sequencing, targeted amplicon sequencing, droplet digital PCR and deep sequencing. Tumour-specific genomic alterations identified in utDNA and ctDNA using these strategies might reflect mutations detected in the primary tumour, or a clonal shift – if new genomic alterations are detected. Similarly, total cell-free DNA (cfDNA) burden and tumour mutational burden (TMB) might reflect patient disease volume and correlate with imaging findings. Lastly, cancer cell fractions (CCFs) and variant allele frequencies (VAFs) might be used to assess minimal residual disease and metastatic burden, whereas copy number variants (CNVs) might be used to assess alterations in specific genes. GA, genomic alteration.

Muscle-invasive and metastatic bladder cancer

TP53 has emerged as the most commonly mutated gene in patients with MIBC, occurring in 48% of tumours, with inactivating mutations being the most frequent *TP53* alterations[20,21]. Additionally, *MDM2* overexpression or amplification, which inhibits p53 signalling through the inhibition of *TP53* transcription, occurs in 6% of patients with MIBC[22]. Other commonly altered genes in patients with MIBC include *MLL2* (27%), *ARID1A* (25%), *KDM6A* (24%), *PIK3CA* (20%), *CDKN1A* (14%), *RB1* (13%) and *ERCC2* (12%)[21]. *MTAP* loss on chromosome 9, which is nearly universally accompanied by *CDKN2A and CDKN2B* loss, has been found in 25% of patients with clinically advanced bladder cancer, and is associated with an adverse prognosis[23]. In a study including 412 tumour samples from patients with MIBC, mutated genes and focal somatic copy number alterations (CNAs) were identified, and unsupervised clustering was used to generate four DNA-based clusters that were characterized by *TP53* and *RB1* mutations, *SOX4* or *E2F3* amplification, mutations in chromatin-modifying genes, and *FGFR3*, *KDM6A* and *STAG2* mutations, respectively[21]. Moreover, in the same study five distinct mutational signatures were described with different molecular drivers: APOBEC-a, APOBEC-b, C>T transition at CpG dinucleotides, POLE, and ERCC2; APOBEC-related mutations accounted for 67% of all single-nucleotide variants[21]. APOBEC cytidine deaminase enzymes are normally involved in deamination of single-stranded RNA (ssRNA), but can cause hypermutation at cytosine bases in exposed ssRNA of tumour cells, leading to cellular proliferation[24]. The high somatic mutation rates observed in patients with MIBC (7.7 mutations/Mb) lead to a reduced responsiveness to immune checkpoint blockade in these patients[25]. In other studies, actionable genomic alterations were present in 30–83% of profiled tumours from patients with MIBC, including mutations in *PIK3CA*, *CDKN2A* and *CDKN2B*, *CCND1*, *FGFR1* and *FGFR3*, *ERBB2*, *BRCA2*, *TSC1*, *HRAS* and *NTRK1* (refs. [26,27]).

Cisplatin-based chemotherapy remains the current standard-of-care for eligible patients with MIBC and metastatic urothelial carcinoma (mUC)[28]. Failure to repair treatment-induced DNA damage has been widely reported as a mechanism of sensitivity to chemotherapy, particularly to the DNA alkylating agent cisplatin[29]. Tumours with deleterious alterations in the DNA damage repair genes *ATM*, *RB1*, *ERCC2* and *FANCC* are unable to repair chemotherapy-induced DNA damage and, therefore, are particularly sensitive to chemotherapy[29–31]. Additionally, some somatic missense mutations found in *ERBB2* were shown to increase ERBB2 susceptibility to phosphorylation and subsequent degradation in response to cisplatin, leading to cell death[32,33]. This evidence is important considering that ERBB2 activation causes proliferation of tumour cells, and this gene can be amplified in malignancies such as breast and urothelial carcinoma[34].

Several efforts have been directed towards the characterization of genomic evolution in response to selective pressure from systemic therapy. In a study in which whole-exome sequencing was carried out on 72 samples from patients with urothelial carcinoma, substantial molecular differences were observed between pre-chemotherapy and post-chemotherapy tumours from the same patient, and much of the post-treatment genomic heterogeneity was ascribed to the activity of APOBEC-induced mutagenesis of selected genes, such as the ABC family of proteins and homologous recombination DNA damage repair genes. In another study, a cisplatin chemotherapy-induced mutational signature characterized by accumulating C>A mutations in patients with MIBC was identified[35]; in this study, high post-chemotherapy genomic heterogeneity was shown to be a prognostic factor for poor overall survival (OS).

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Table 1 | Studies analysing ctDNA in urothelial carcinoma

Patients	Sequencing method	Genomic alterations assessed	Ref.
29 (NMIBC), 7 (MIBC)	qPCR	Microsatellite analysis	7
32 (NMIBC)	Spectrophotometry	Plasma DNA concentration	103
12 (NMIBC)	ddPCR	One to six personalized assays per patient	67
22 (mUC)	CAPP-seq	Mutations in *TP53*, *TERTp*, *ARID1A*, *FGFR2*, *FGFR3*	125
216 (NMIBC), 27 (MIBC)	ddPCR	Mutations in *PIK3CA* and *FGFR3*	75
17 (MIBC), 2 (UTUC)	TAm-seq	Mutations in *TP53*, *PIK3CA*, *FGFR3*, *NFE2L2*	104
24 (NMIBC), 49 (MIBC)	MLPA	43 CNVs in *KLF5*, *ZFH3* and *CDH1*	126
14 (MIBC), 37 (mUC)	CAPP-seq	ctDNA fraction; mutations in *ERBB2* and *TP53*	117
369 (mUC)	CAPP-seq	Alterations in 73 genes assessed	36
50 (MIBC), 10 (mUC)	ddPCR	One to six personalized assays per patient	127
29 (mUC)	CAPP-seq	MAFs of somatic variants	128
84 (NMIBC), 9 (MIBC)	qPCR	Mutations in the *TERT* promoter	74
36 (MIBC)	ddPCR	Mutations in *FGFR3*	129
68 (MIBC)	WES, UDTS	16 highly ranked somatic mutations	95
20 (NMIBC), 5 (MIBC)	CAPP-seq	Alterations in 71 genes assessed	70
124 (mUC)	CAPP-seq	Alterations in 73 genes assessed	100
47 (MIBC)	ddPCR	Total cfDNA, short and long fragments	130
5 (UTUC), 39 (UCNOS)	CAPP-seq	600-gene panel	131
2 (NMIBC), 18 (MIBC)	ddPCR	Mutations in *PIK3CA* (*PIK3CA*E542K), *TP53* and *TERTp*	105
4 (NMIBC), 39 (MIBC)	MLPA	CNVs in *MYC*, *CCND1*, *ERBB2* and *CCNE1*	99
24 (NMIBC), 23 (MIBC)	cf-SUPER	22 genes assessed	76
1 (mUC)	ddPCR	Mutations in *MLH1*, TMB	132
16 (mUC)	PCR–NGS	ctDNA fraction; mutations in *TP53*, *TERT* and *ERBB2*	133
13 (UCNOS)	CAPP-seq	324 genes assessed	134
135 (mUC)	PCR–NGS	Mutations in 16 genes	114
581 (UCNOS)	PCR–NGS	ctDNA positivity and clearance	113
104 (mUC)	WES, NGS	Mutations in *FGFR3*, *ERCC2* and *ERBB2*	97
82 (NMIBC)	CAPP-seq	TMB, mutations in *FGFR3*, *PIK3CA*, *ERBB3* and *HRAS*	98
48 (NMIBC), 11 (MIBC)	CAPP-seq	Mutations in *FGFR3* and ERBB2; cfDNA abundance	68
10 (UTUC)	CGP	Mutations in *FGFR3*	42
53 (mUC)	PCR–NGS	Aggregate VAFs	26

CAPP-seq, cancer personalized profiling by deep sequencing; cfDNA, cell-free DNA; cf-SUPER, cell-free single-molecule unique primer extension resequencing; CGP, comprehensive genomic profiling; CNVs, copy number variants; ctDNA, circulating tumour DNA; ddPCR, droplet digital PCR; MAFs, mutation allele frequencies; MIBC, muscle-invasive bladder cancer; MLPA, multiplex ligation-dependent probe amplification; mUC, metastatic urothelial carcinoma; NGS, next-generation sequencing; NMIBC, non-muscle-invasive bladder cancer; qPCR, quantitative PCR; TAm-seq, targeted amplicon sequencing; TMB, tumour mutational burden; UCNOS, urothelial carcinoma not otherwise specified; UDTS, ultra-deep targeted sequencing; UTUC, upper tract urothelial carcinoma; VAF, variant allele frequency; WES, whole-exome sequencing.

The diversification in genomic alterations induced by chemotherapy might not be evident from biopsies obtained from the primary untreated tumour, and serial metastatic biopsies required to accurately track the evolving tumour mutational landscape might not be feasible in these patients, who are generally elderly and frail with a substantial disease burden. Conversely, the ctDNA mutational profile might be obtained through non-invasive serial liquid biopsies to track treatment-induced changes in genomic alterations, and also to detect new targets for the treatment of patients with resistant tumours.

Upper tract urothelial carcinoma

UTUC shares many genomic traits with bladder cancer, but remains a distinct clinical entity. The most common alterations found in patients with UTUC include mutations within the *TERT* promoter (43.4%), *KMTD2* (40.7%), *TP53* (39.7%), *CDKN2A* (36.1%) and *FGFR3* (33.2%)[36–45]. UTUC has a lower TMB than bladder cancer, with median TMB ranging between 5.2 and 10.9 mutations/Mb for high-grade UTUCs[41,42]. However, the median number of mutations per tumour was significantly higher in patients with Lynch syndrome-associated UTUCs than in patients with sporadic-type UTUC (58 versus 6 genomic alterations per tumour, respectively)[46].

Similar to what has been observed in bladder cancer, frequent *TP53* mutations have been described in high-grade[40] and/or non-organ-confined UTUC tumours[47]. *TP53* and *MDM2* alterations have been shown to be associated with an increased risk of distant metastases following radical nephroureterectomy and increased rates of UTUC-related deaths[47]. In several studies, alterations in the *FGFR3* signalling pathway were shown to be common in patients with low-grade[37,43,47], non-invasive[38,44] UTUC. Recurrent gene fusions involving *FGFR3* and *TACC3* have also been reported in patients with UTUC[40,44]. *FGFR3* alterations also frequently co-occur with mutations in the chromatin-modifying gene *KMT2C*[43,44].

In studies in which specific genomic alterations from tumours with a similar genomic landscape were clustered together, the molecular subtyping of bladder cancer described in The Cancer Genome Atlas (TCGA) was used for data analysis[38,44]. Hierarchical clustering of whole-exome sequencing profiles of 199 tumour samples from treatment-naive patients with UTUCs resulted in the identification of five different gene expression profiles with discrete tumour location, histology and clinical outcomes: hypermutated, with high TMB; *TP53*-mutated and *MDM2*-mutated; *FGFR3*-mutated; *RAS* hotspot-mutated; and triple-negative (without *TP53*, *FGFR3* or *RAS* mutations)[38]. This classification has important prognostic relevance in UTUC, as *FGFR3*-mutated disease was shown to correlate with favourable survival, whereas *TP53*-mutated/*MDM2*-mutated and triple-negative subtypes were associated with poor cancer-specific survival[37]. Identification of these genomic markers and tumour subtypes using liquid biopsies might facilitate risk stratification and drive clinical decision-making for selecting optimal treatment strategies based on the UTUC subtype.

cfDNA biology and sequencing technology

cfDNA is fragmented DNA found within the extracellular compartment of the blood (or other bodily fluids), which is released by cells through apoptosis and necrosis[48] (Fig. 1). cfDNA concentration has been shown to be increased in a variety of physiological and pathological conditions including acute trauma[49], cerebral infarction[50], exercise[51], infection[52] and cancer[53]. In healthy individuals, the majority of cfDNA within the plasma is released from haematopoietic cells[54]. Genetic and epigenetic modifications found in cfDNA molecules closely resemble the

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Table 2 | Studies analysing utDNA in urothelial carcinoma

Patients	Sequencing method	Genomic alterations assessed	Ref.
29 (NMIBC), 7 (MIBC)	qPCR	Microsatellite analysis	7
51 (UCNOS)	qPCR	cfDNA concentration; mutations in *HER2* and *MYC*	135
220 (UTUC)	qPCR	Mutations in the *TERT* promoter	136
32 (NMIBC)	Spectrophotometry	Plasma DNA concentration	103
12 (NMIBC)	ddPCR	One to six personalized assays per patient	67
47 (NMIBC), 26 (MIBC)	qPCR	*TopoIIA* mutations in cfDNA	137
9 (NMIBC), 14 (MIBC)	Oncoscan	74 somatic mutations tested, CNVs	71
5 (UCNOS)	qPCR	Mutations in *MYC*, *BCAS1*, *HER2* and *AR*	138
216 (NMIBC), 27 (MIBC)	ddPCR	Mutations in *PIK3CA* and *FGFR3*	75
17 (MIBC)	TAm-seq	Mutations in *TP53*, *PIK3CA*, *FGFR3* and *NFE2L2*	104
50 (MIBC), 10 (mUC)	ddPCR	One to six personalized assays per patient	127
58 (NMIBC), 19 (mUC)	qPCR	Mutations in *IQGAP3* and *TOPIIA*	139
9 (MIBC)	Deep sequencing	Mutations in *MDM2*, *ERBB2*, *CCND1*, *CCNE1* and *RB1*	134
104 (UCNOS)	ddPCR	Mutations in *TERT* sequences	140
84 (NMIBC), 9 (MIBC)	qPCR	Mutations in *TERT* promoter	74
101 (NMIBC), 36 (MIBC)	ddPCR	*FGFR3* mutations	129
37 (NMIBC), 9 (MIBC)	Methylation	Hypomethylations, CNAs	90
102 (NMIBC), 16 (MIBC)	CAPP-seq	Mutations in *TERT*, *PLEKHS1* and *TP53*	79
484 (NMIBC), 43 (MIBC)	Multiplex PCR–NGS	Mutations in *TERT*, *FGFR3*, *PIK3CA* and *TP53*	141
56 (UTUC)	ddPCR	Mutations in the *TERT* promoter and *FGFR3*	142
20 (NMIBC), 5 (MIBC)	CAPP-seq	Mutations in 71 genes assessed	70
43 (NMIBC), 10 (MIBC)	Multiplex PCR–NGS	*TERT* promoter MAFs	143
103 (NMIBC)	RT-PCR	*IQGAP3:BMP4* ratio	144
81 (NMIBC), 68 (MIBC)	RT-PCR	*IQGAP3:BMP4* ratio	89
16 (UTUC), 49 (UCNOS)	sWGS	*CAN* mutations in cfDNA	145
28 (NMIBC), 8 (MIBC)	ddPCR	Mutations in the *TERT* promoter and *FGFR3*	142
12 (NMIBC), 2 (MIBC)	Methylation	Mutations in *GHSR*, *SST* and *MAL*	146
99 (UCNOS)	ddPCR	Mutations in the *TERT* promoter	14
26 (UTUC)	Methylation	Sensitivity in diagnosis of low-risk disease	147

Patients	Sequencing method	Genomic alterations assessed	Ref.
42 (NMIBC), 50 (MIBC)	RT-PCR	Mutations in *TERT*, *FGFR3*, *TP53*, *PIK3CA* and *KRAS*	72
2 (NMIBC), 18 (MIBC)	ddPCR	Mutations in *PIK3CA* (*PIK3CA*^E542K), *TP53* and *TERT* promoter	105
156 (NMIBC)	MASO-PCR	*FGFR3* mutations	148
98 (NMIBC)	RT-PCR	Mutations in *TERT*, *FGFR3* and *KRAS*	149
24 (NMIBC), 23 (MIBC)	PCR–NGS	22-gene panel	76
32 (MIBC)	CAPP-seq	TMB, mutations in *TERT*, *TP53* and *ARID1A*	150
25 (NMIBC), 9 (MIBC)	ddPCR	Mutations in the *TERT* promoter	73
10 (UTUC)	CGP	*FGFR3* mutations	42
82 (UTUC)	Methylation	15 methylation biomarkers	81
12 (UTUC)	Multiplex PCR–NGS	Mutations in *FGFR*, *TERT* and *PIK3CA*	85
48 (NMIBC), 11 (MIBC)	CAPP-seq	Mutations in *FGFR3* and *ERBB2*; cfDNA abundance	68
37 (NMIBC), 9 (MIBC)	Methylation, NGS	Jagged end index	151

CAPP-seq, cancer personalized profiling by deep sequencing; cfDNA, cell-free DNA; CGP, comprehensive genomic profiling; CNAs, copy number alterations; CNVs, copy number variants; ddPCR, droplet digital PCR; MAFs, mutation allele frequencies; MASO-PCR, mutated allele-specific oligonucleotide PCR; MIBC, muscle-invasive bladder cancer; mUC, metastatic urothelial carcinoma; NGS, next-generation sequencing; NMIBC, non-muscle-invasive bladder cancer; qPCR, quantitative PCR; RT-PCR, PCR with reverse transcription; sWGS, shallow whole-genome sequencing; TAm-seq, targeted amplicon sequencing; TMB, tumour mutational burden; UCNOS, urothelial carcinoma not otherwise specified; utDNA, urinary tumour DNA; UTUC, upper tract urothelial carcinoma.

genomic profile of the cell of origin[2]. Additionally, cfDNA has a short half-life (16 min to 2.5 h) within the circulation[55] and, therefore, is an ideal 'real-time' peripheral genomic fingerprint of the cell of origin.

The introduction of digital PCR (dPCR) technology and modified versions of this technology using beads in emulsion and flow cytometry enabled the quantification of rare mutant fragments as well as variant allele fractions (VAFs) of ctDNA in patients with various stages of cancer[56]. These assays are quantitative and highly sensitive, but the multiplexing capacity is limited by the need to design primers that are specific for a defined mutation or target locus and can differentially bind mutant and wild-type alleles. Thus, dPCR assays are best suited for investigating small numbers of mutations, such as cancer hot-spot mutations.

With the advent of next-generation sequencing (NGS)-based technologies, broad scale interrogations of the genome became possible. Initially, deep sequencing of multiple genes using panels of tagged amplicons enabled researchers to identify and monitor multiple tumour-specific mutations in a single assay[57]. Not long after the introduction of NGS, various chromosomal and genomic alterations were detected through massive parallel whole-genome sequencing (WGS) of plasma cfDNA[58]. Hybrid capture sequencing, in which hybridization between oligonucleotide baits and the complementary target DNA occurs in solution, was used as a non-invasive method to obtain a broad analysis of the tumour genomic mutational profile[58]. Regions of sequencing might range from individual exons of interest (a few kilobase) to the entire exome (~50 Mb). Rare mutations can be detected

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through ready-to-purchase gene sequencing panels with allele fractions down to ~1%[59]. Reduction in the background error rates through molecular barcoding (a method used for genomic identification) or running multiple replicates can further increase sequencing sensitivity to detect ctDNA, with allele fractions as low as 0.1%[60].

Lastly, the sensitivity of NGS in detecting mutant alleles in ctDNA and utDNA is influenced by whether the patient's primary tumour has already been profiled. The sequencing of a primary tumour site enables the creation of a specific DNA library and is a tumour-informed approach, as opposed to tumour-naive approaches. Bespoke assays used in the tumour-informed approach can be used to detect known patient mutations above the background error rate, rather than to identify de novo mutations[57]. With this approach driven by genomic analysis of a tumour, sequencing-based assays can be used as alternatives to dPCR as quantitative tools for ctDNA measurement and monitoring. Furthermore, ctDNA quantification can be expressed either as variant allele concentration (copies per millilitre) or VAF.

Gene amplifications and deletions can be identified through shallow-depth sequencing of the tumour whole genome (~0.1× coverage) and comparison of the relative number of reads among equally sized genomic regions[61]. Using this shallow WGS (sWGS) technique, VAF of 5–10% can be detected; moreover, sWGS was used to generate a genome-wide copy number profile of the primary urothelial tumour to further understand cancer clonal evolution[61]. Combining sWGS with the highly sensitive targeted dPCR technology, copy number variants (CNVs) were accurately detected with an average allelic imbalance as low as 0.5%[62].

Practical challenges exist in the study of ctDNA within the urine. First, sample volumes are typically larger than plasma, increasing the difficulty of cfDNA extraction. A resin-based cfDNA extraction protocol was described as a strategy to analyse large sample volumes maintaining an extraction performance comparable with that of commercial kits[56]. In this study, urine cfDNA concentration was shown to remain stable for at least 7 days at 4 °C in the presence of EDTA. Furthermore, enzymatic fragmentation induced higher DNA recovery than acoustic shearing, increasing the deduplicated sequencing depth by about twofold, which resulted in the detection and recognition of genetic material expressed at low abundances[63]. Lastly, the range of cfDNA fragment sizes was wider in urine than plasma samples, suggesting that appropriate protocol modifications will be necessary for downstream DNA library preparation and bioinformatics analysis pipelines[57].

In summary, advances in techniques for genomic sequencing of peripheral blood and urine have facilitated ctDNA and utDNA detection and identification.

Feasibility of liquid biopsy

Both utDNA and ctDNA fall under the umbrella of 'liquid biopsy', which consists of sampling a fluid as a surrogate for tissue sampling. In patients with urothelial carcinoma, a unique opportunity to capture cfDNA as a liquid biopsy exists, as ctDNA can be detected from the circulating blood, whereas utDNA might reflect direct shedding of material from malignant cells in the urothelium or filtration from peripheral blood through the glomerulus[64,65].

Plasma versus urine

To date, peripheral blood has been the most commonly used source of liquid biopsy samples, owing to a thorough understanding of the stability of cfDNA in plasma, optimal isolation and collection protocols, as well as commercially available containers pre-prepared with standard fixatives to favour maximal DNA stability[66]. Urine might be an alternative potentially rich source of tumour-associated cfDNA, particularly in the context of urothelial carcinoma. Advantages of utDNA include the ease of acquisition and relative lack of contaminants.

The feasibility of using urinary ctDNA in comparison with that of plasma ctDNA for the detection of urothelial cancer was assessed in two important studies. In the first study[67], a thorough genomic analysis of tumour tissue, as well as longitudinally collected plasma and urinary cfDNA were carried out in patients with progressive and recurrent NMIBC. Genomic alterations found within the tumour tissue were used as a reference, and one to six personalized assays were developed to identify genomic alterations in cfDNA from liquid biopsy samples with high sensitivity (1/6,000 background copies). ctDNA was detectable in 10 of 12 plasma samples (83%), whereas utDNA was detectable in 101 of 116 urine samples (87%). Using both plasma and urine, cfDNA detection rates were higher among patients with progressive NMIBC than among patients without disease stage progression. Some crucial observations about tumour clonality emerged from this study: increased genomic heterogeneity was present in tumour biopsy samples collected metachronously in patients with progressive NMIBC. The identification of various genomic alterations between metachronous tumours indicates that a wide array of dPCR probes are necessary to encompass all genomic alterations from these patients, and different variants were released into the circulation at different collection time points, indicating possible clonal evolution. Furthermore, baseline utDNA levels in patients with progressive disease (1,282 copies/ml) were higher than the levels found in patients with recurrent NMIBC (31 copies/ml), suggesting that utDNA levels might have a prognostic role at the time of NMIBC diagnosis.

In another study including 59 patients with primarily early stage bladder cancer[68], the advantage of using urine over plasma as a source of liquid biopsy samples was shown. In the analysis of cancer cell fraction, metrics such as maximum somatic allelic frequency, total number of mutations and TMB assessed through NGS were compared among tumour, ctDNA and utDNA samples; the genomic landscapes in tumour tissues and urine were highly similar, but distinct from those in plasma. utDNA was abundantly expressed in patients with high-grade or high-stage tumour, carcinoma in situ (CIS), increased Ki-67 proliferation index, or concomitant haematuria. Detection of utDNA achieved a sensitivity of 86.7% and specificity of 99.3% for diagnosing bladder cancer. Other potential clinical applications of utDNA sequencing include prediction of tumour recurrence as well as the identification of actionable mutations for targeted therapy.

Both utDNA and plasma ctDNA offer detectable tumour-derived nucleic acids, but consideration should be given to the tumour population tested and the clinical question posed. In tumours with reduced invasive properties and a low risk of metastasis (such as NMIBC), utDNA might be the most relevant and appropriate assay. Conversely, ctDNA might be the most valid assay to reflect the overall tumour burden, the presence of micrometastatic disease, and clonal evolution that takes place during treatment.

Urine supernatant versus cell pellet

The predominance of studies in which utDNA is assessed in urothelial cancer supports urine as the fluid of choice for liquid biopsy samples in this type of cancer. Within utDNA extraction, genomic profiles from cfDNA in the urine supernatant and from cell pellets after centrifugation have been compared in different studies. cfDNA is mostly found within the urine supernatant, whereas pellets primarily consist of exfoliated normal and cancer cells, in addition to immune cells and cellular debris[69].

Results from de novo targeted sequencing of a 71-gene panel in utDNA samples from patients with bladder cancer showed similar variant allelic detection rates and median allelic frequencies in urine supernatant cfDNA and cell pellet DNA[70]. Moreover, high correlation between VAFs detected in the two compartments was shown ($R^2 = 0.74$). Conversely, in another study[71] in which cell pellet and urine supernatant were referenced to bulk sequencing data from formalin-fixed paraffin-embedded tumour samples, cfDNA in urine supernatant enabled the identification of tumour genomic alterations with higher sensitivity than DNA in cell pellets, with an area under the curve (AUC) of 0.94. Genomic mutations identified included clinically actionable mutations. Interestingly, some mutations in *PIK3CA* and *FGFR3* were frequently found in urinary cell pellet DNA collected from patients without cancer, raising concerns for false-positive results using cell pellet DNA[72]. Results from these studies show that urine supernatant seems to be the ideal liquid biopsy source of DNA to detect urothelial cancer.

Implementation of utDNA to diagnose urothelial cancer

Current knowledge about the most prevalent mutations across patients with upper and lower tract urothelial cancers has been used to design low-cost, high-throughput screening assays to diagnose these cancers. These utDNA assays range from the use of a single commonly mutated gene (*TERT* promoter[73,74]) to a panel of mutations associated with urothelial cancer[75–77]. Using dPCR-based approaches, VAF detection as low as 0.01% has been reported in patients with bladder cancer[76]. Many of these mutations are often found in patients with low-grade bladder cancer; thus, these assays consistently outperform urine cytology, showing sensitivity and specificity above 80–90%[73–76]. Conversely, urine cytology is associated with a notoriously poor detection rate for urothelial carcinoma, with sensitivity ranging from 40% to 76%[78].

Perhaps the most comprehensive effort at ctDNA biomarker identification consisted of the development of a hybrid capture method called urinary cancer personalized profiling by deep sequencing (uCAPP-seq)[79]. A panel of 460 genes commonly mutated in bladder cancer was constructed based on TCGA data and applied to 118 urine and 60 tumour samples from 130 patients with bladder cancer[79]. A median of six mutations per patient were identified, with a 66.7% concordance in genomic alterations between tissue and urine samples, which established the assay's feasibility. The assay was then used on urine samples collected from 67 healthy adults and 118 patients with early-stage bladder cancer, either before treatment or during surveillance[79]. Using this urinary assay, genomic patterns previously described in bladder cancer were successfully confirmed. Prominent genomic alterations included *TERT*, *PLEKHS1*, *TP53*, *KDM6A* and *FGFR3* (ref. [79]). uCAPP-seq also enabled the identification of early-stage bladder cancer and the prediction of recurrence during surveillance[79]. When performed with a tumour-naive approach, the assay had a sensitivity of 77.5% for the detection of Ta tumours and 100% for higher-stage tumours; detection rates of 96% and 72.4% were observed for high-grade and low-grade lesions, respectively[79]. In the surveillance setting, uCAPP-seq enabled the detection of 100% of recurrence instances also detected through urine cytology, and 73.9% of recurrences that were missed by cytology evaluation. Moreover, detection of utDNA preceded clinical disease recurrence in 92% of patients by a median of 2.7 months[79]. These preliminary results need validation, but might have major implications in the screening and surveillance of patients with bladder cancer.

In other studies, DNA methylation markers were used for urothelial cancer diagnosis. Bladder EpiCheck is a non-invasive PCR-based assay to detect methylation at 15 genomic loci[80]. In a multicentre study in which this assay was used to monitor recurrence in patients with NMIBC, an overall sensitivity of 68.2% and a specificity of 88.0% were observed[80]. This test was also used to detect UTUC using urine samples collected through selective ureteral catheterization, and provided much higher sensitivity and specificity (97.4% and 100%, respectively) than urine cytology (58.9% and 96.0%, respectively)[81]. Additional methylation assays include a 23-marker multiplex urinary methylation assay named urine tumour DNA methylation MAssARRAY (utMeMA), which was developed through comparison of tumour and normal tissue methylation profiling using samples from both the Sun Yat-sen Memorial Hospital database and TCGA[82]. utMeMA was used in a large cohort of 142 patients with bladder cancer and 171 healthy individuals, and an AUC of 0.92 was achieved[82]; utMeMA was then validated in a prospective, multicentre, blinded study including 175 patients with bladder cancer and showed a sensitivity and specificity in diagnosing bladder cancer of 91.7% and 86%, respectively. Additionally, positive utMeMA assays were shown to positively correlate with high-grade, advanced stage and/or multifocal disease, indicating that patients in this clinical setting have an increased burden of methylated genomic regions.

To date, no head-to-head comparison between utDNA mutation and methylation assays has been carried out to evaluate the accuracy of these assays in the diagnosis of urothelial cancer. However, the two tests might be complementary, and a promising strategy could be to combine the two assays to be used in tandem for diagnostic purposes. In a multicentre study including 200 patients with haematuria, the diagnostic yield of mutations in *FGFR3*, *TERT* and *HRAS* and the methylation status of *OTX1*, *ONECUT2* and *TWIST1* in urine DNA for the diagnosis of bladder cancer was evaluated[83]. Using a model in which mutations and methylation statuses were combined with age, an AUC of 0.96 (95% CI 0.92–0.99) with 93% sensitivity and 86% specificity was achieved. Considering the high proportion of bladder cancers diagnosed from a haematuria workup[84], implementing this assay was estimated to lead to a reduction of ~80% in the number of unnecessary cystoscopies[83]. In a similar study in which a logistical regression model combining patient age, *TERT* mutation status and *ONECUT2* methylation levels in utDNA was used in the setting of UTUC, a sensitivity of 94.0%, a specificity of 93.1% and an AUC of 0.957 were achieved in diagnosing UTUC, indicating that complementary methylation and utDNA are promising diagnostic tools in urothelial carcinoma of both the upper and lower urinary tract[85].

Qualitative and quantitative measures of cfDNA characteristics have also been linked to the presence of urothelial cancer. For instance, microfluidics-based platforms were used to assess the concentration and range of ctDNA fragment size in a cohort of 69 patients with urothelial cancer and matched healthy individuals[71]. In patients with cancer, concentrations of plasma ctDNA were elevated, whereas fragment lengths were shortened. Additionally, plasma ctDNA fragment size tended to shorten along with increasing pathological stage ($P < 0.001$) and was associated with increasing levels of inflammatory markers such as C-reactive protein and neutrophil-to-lymphocyte ratios. Lastly, reduced ctDNA fragment size predicted worse OS. These findings indicate that although ctDNA detects genomic alterations of the primary tumour peripherally, further qualitative characteristics of ctDNA, such as fragment length, hold promise for correlative studies.

In summary, utDNA can be used to assess a single mutated gene versus a panel of commonly mutated genes in patients with urothelial carcinoma in bespoke or tumour-naive settings. utDNA has consistently outperformed urine cytology and can improve current bladder cancer diagnosis, staging and surveillance protocols.

Review article

Bench to bedside

The variety of analytical methods proposed for using cfDNA in the diagnosis of urothelial cancer is promising, but these methods are still far from clinical implementation. Careful consideration of the current diagnostic, prognostic and predictive shortfalls in the management of urothelial cancer, as well as an improved understanding of how the application of liquid biopsy might replace or supplement the current gold standard diagnostic techniques is needed before liquid biopsy can move from the bench to the bedside. Furthermore, the understanding of urothelial cancer biology and molecular therapeutics is growing, and granular genomic data extrapolated from cfDNA studies will increasingly support treatment selection and disease monitoring. Thus, future clinical investigations involving cfDNA assays should aim to eradicate disease and/or prolong survival, rather than less relevant molecular end points such as genomic landscape or mutational profiles alone. Several phases of clinical practice are likely to be transformed by the adoption of liquid biopsies (Box 1).

Cancer diagnosis

The rationale for screening is to detect cancer in early stages, when the probability of disease cure is increased. Evidence has shown the feasibility and benefit of screening in urothelial cancer[86,87], but no current protocol exists owing to the relative rarity of the disease and the very high costs associated with screening for urothelial carcinoma Conversely, the diagnosis is often derived from the work-up of patients presenting with haematuria, the most common symptom of urothelial cancer[84]. Unfortunately, diagnostic yield is low even among these high-risk patients, as haematuria might be indicative of other non-malignant

genitourinary conditions[88]. The lack of consensus on which patients should undergo mandatory haematuria workup, and the invasiveness of the screening procedures are a burden to both patients and the urological health-care system. Currently available non-invasive screening tools such as urine cytology are notoriously unreliable, with low sensitivity (40–76%) for cancer detection[78]. Thus, screening through accurate non-invasive technologies such as utDNA assays might have an improved risk–benefit balance and transform the management paradigm of urothelial cancer.

Results from a study in which utDNA gene expression levels and genomic ratios were assessed in patients with haematuria showed a sensitivity of 71% and a specificity of 88.6% for bladder cancer detection, supporting the potential of utDNA as a screening tool for bladder cancer[89] Similarly, the assessment of synergistic CNAs and DNA methylation in utDNA samples from patients with haematuria enabled detection of bladder cancer in the all-comers population with a sensitivity of 94%, whereas the sensitivity for low-grade NMIBC detection was 84%[90]. In UTUC, utDNA analysis has shown promising diagnostic results in patients with haematuria, with a specificity as high as 95%[85]. These results suggest a potential future role of utDNA in the assessment of patients with haematuria, which might improve risk stratification and drive a personalized and streamlined workup of these patients.

Staging and prognosis

Current prognostic stratification of patients with urothelial cancer relies on tumour staging, primarily carried out after transurethral resection of bladder tumour (TURBT) in patients with NMIBC and radical extirpative surgery in patients with MIBC or UTUC[91]. In a correctly

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performed TURBT, the muscularis propria of the bladder is sampled, and also all pre-existing disease should be resected. However, this idealized standard is often not met in clinical practice, as the disease is under-staged in up to 25% of patients with invasive cancers[92], and residual tumours are found in up to 20% of patients following primary resection[93]. When tumour extends beyond the bladder wall, characterization by cross-sectional imaging is often difficult, as the tumour resembles oedema and inflammation caused by TURBT or prior intravesical therapy[94]. These issues related to tumour staging could be potentially addressed with the use of ctDNA, as utDNA analysis can help detect residual and aggressive disease within the bladder, whereas plasma ctDNA can be used to identify the presence of extravesical disease. Moreover, genomic alterations or patterns of alterations detected within the urinome and circulome might have prognostic value and offer risk stratification opportunities.

Increased levels of ctDNA were shown to correlate with disease burden and predicted disease recurrence, progression and worse outcomes in patients with bladder cancer[67]. In a proof-of-concept study, cfDNA levels in patients with progressive NMIBC or recurrent NMIBC were compared; higher cfDNA levels were observed in both plasma and urine samples collected at baseline from patients who eventually progressed to MIBC than patients who did not experience disease progression[67]. These results indicate that cfDNA levels predict clinical progression, suggesting that cfDNA can be used as a predictive biomarker for disease worsening. In a subsequent study from the same group, in which a non-tumour-informed selective hotspot mutation assay was used, high utDNA levels were shown to be associated with increasing tumour burden, grade and European Organisation for Research and Treatment of Cancer risk score [75]. In another study[68], utDNA cancer cell fraction, median variant allele frequency, maximum somatic allele frequency, total number of mutations and TMB were shown to predict bladder tumour recurrence. In this study, high utDNA levels also correlated with the presence of high-grade, late-stage CIS, and increased Ki-67 proliferation index, indicating that utDNA might help identify patients with bladder cancer who are at high risk of recurrence and progression.

In a study including 68 patients with clinically localized MIBC undergoing neoadjuvant chemotherapy[95], tumour-informed patient-specific somatic mutations identified through ultra-deep sequencing were used to assess the prognostic and predictive properties of ctDNA. Samples were collected after TURBT and before chemotherapy (when the diagnosis was established), after chemotherapy and before cystectomy, and following surgery during surveillance. Detectable (positive) ctDNA following initial diagnosis and at the completion of chemotherapy was shown to be highly prognostic for disease recurrence. Moreover, negative ctDNA following chemotherapy predicted pathological complete response at the time of radical cystectomy with 100% accuracy[95]. Taken together, these results indicate that ctDNA assessment is effective for risk stratification, therapeutic response monitoring and detection of early relapse in MIBC.

The prognostic role of ctDNA has also been assessed in patients with mUC. In a study involving 978 patients with 16 different malignancies (including mUC) receiving immunotherapy treatment with durvalumab with or without tremelimumab, a high pretreatment ctDNA VAF was associated with reduced OS, but not reduced objective response, suggesting a prognostic role of VAF[96]. On-treatment reductions in VAF, but not in other prognostic variables (such as Eastern Cooperative Oncology Group score, metastatic burden and smoking status), were associated with increased progression-free survival (PFS), OS and objective response rate, suggesting that ctDNA dynamics are predictive of immunotherapy benefit. Overall, pretreatment plus on-treatment VAF predicted long-term survival, in turn enabling early differentiation of responders to therapy.

In a study including 71 patients with mUC undergoing first-line systemic treatment[97], low baseline ctDNA fraction within total cfDNA was associated with improved OS (HR 3.1, 95% CI 1.3–7.5, in patients with high ctDNA). Similar findings were obtained in another study in which a low ctDNA VAF (≤0.2%) was shown to be associated with increased OS (HR 0.31, 95% CI 0.11–0.90), even in a multivariate analysis incorporating other clinical prognostic variables[26].

In addition to quantitative measures of ctDNA, intratumour heterogeneity can also be used for prognostic purposes[98]. In a study including patients with NMIBC undergoing TURBT followed by immunotherapy[98], the investigators showed that detecting subclonal mutations within plasma ctDNA is feasible, particularly in patients with high-grade and high-stage NMIBC. For example, ctDNA was detectable in 85.3% of patients with high-grade T1 disease and in 52.1% of patients with high-grade Ta disease ($P < 0.01$). In this study, a combination of ctDNA levels and clonal diversity was used to calculate a molecular tumour burden index that was shown to increase prediction of disease recurrence. In studies in which specific alterations found in patients with MIBC were assessed in ctDNA samples, the presence of CNVs in *ERBB2* was associated with a locally aggressive tumour phenotype, whereas CNVs in *TOP2A* conferred an increased risk of disease recurrence after surgical extirpation[99]. In another study, *BRCA1* and *RAF1* alterations in plasma ctDNA were shown to be associated with reduced OS in a cohort of patients with advanced urothelial cancer and mUC[100].

The clinical staging methods currently available for UTUC often lead to an under-staging of the disease secondary to technical and instrumental challenges[101]; thus, the application of ctDNA to improve clinical staging might be particularly relevant in this disease setting. Similar to bladder cancer, some gene mutations can be observed with increased frequency in UTUC of a specific grade or stage. For instance, a higher frequency of *TP53* mutations and lower frequency of *FGFR3* and *PIK3CA* mutations were observed in DNA from urine pellets from patients with high-grade UTUC than in urine pellets from patients with low-grade disease[85]. In a prospective study in which plasma samples collected before surgery from patients with muscle invasive UTUC were analysed[102], a 90% concordance was shown between mutations found in the tumour tissue (through parallel sequencing) and mutations found in plasma ctDNA. Using a non-tumour-informed panel sequencing assay, the investigators found that ctDNA shows promising sensitivity and specificity (71.4% and 100%, respectively) in the detection of muscle-invasive or non-organ-confined UTUC.

In summary, utDNA and ctDNA seem to fill complementary roles in the risk stratification of patients with non-muscle-invasive and muscle-invasive urothelial cancers. utDNA reflects the tumour genomic landscape and can accurately reflect molecular and genomic features of the primary tumour. In NMIBC, utDNA has shown higher sensitivity than ctDNA in the detection of index tumours and recurrences, and should be prioritized over ctDNA[67,68,70,103]. Conversely, plasma ctDNA can serve as a biomarker for the presence of micrometastatic disease, which could be used either before or after treatment. Considering this evidence, utDNA seems to be an intuitively better resource than plasma ctDNA in the management of patients with NMIBC, whereas the combination of ctDNA and utDNA would be more helpful than either assay alone in MIBC[95,104,105]. Considering these insights, several studies have been carried out in which ctDNA was used for the detection of minimal residual disease (MRD).

Review article

Minimal residual disease monitoring

Existing clinical and imaging modalities such as cytology and cross-sectional imaging used for oncological surveillance are not particularly sensitive and might be associated with a substantial lag time in diagnosing recurrent lesions. Tumour burden frequently becomes overwhelming at the time of the confirmed clinical diagnosis, reducing the efficacy of any attempted salvage therapies. Thus, the use of ctDNA as a precocious biomarker is an attractive strategy to predict cancer recurrence[106]. Results from studies including patients with many tumour types have shown the clinical validity of ctDNA in detecting MRD, with a lead time of 3–11 months before clinical or radiographic relapse[107,108].

Overall, two prerequisites must be fulfilled for MRD detection to lead to clinical benefit: MRD must be detected with high sensitivity and specificity in patients with low disease burden, and effective treatments must be available for durable eradication of the disease. In the TRACERx study, a tumour volume of 0.034 cm³ (spherical nodules with diameter of 4 mm) was shown to equate to a plasma VAF of 0.00018%[109]. Detecting ctDNA at such low levels requires prior knowledge of mutations within the tumour, increasing read depth with ultra-deep sequencing and advanced error suppression techniques[110]. Only with these optimizations can MRD detection with these approaches outpace detection by classic imaging modalities.

Enhancing sequencing methods of cfDNA can be used in the treatment of both NMIBC and MIBC. In a study in which the utMeMA assay was used on urine samples collected from patients with NMIBC before re-TURBT[82], utDNA levels were shown to be elevated in patients with residual tumours, which led to a correct diagnosis in 93% of the patients. Conversely, only 64% and 27% of recurrences were detected with UroVysion fluorescence in situ hybridization (FISH) and urine cytology, respectively[82]. These findings suggest that utDNA might outperform currently available urine assays such as FISH and cytology. Similar results from another study showed that recurrent NMIBC was successfully diagnosed in 91% of the patients using a bespoke uCAPP-seq assay[79], and the vast majority of molecular diagnoses preceded clinical progression. Similarly, in another study, uCAPP-seq was used to detect MRD in patients with MIBC undergoing radical cystectomy, and MRD at the time of surgery was detected with 81% sensitivity and 81% specificity[111]. Conversely, when utDNA was incorporated in the trial protocol in pilot studies including patients undergoing bladder preservation with chemoradiation[112], MRD was detected with lower sensitivity than that obtained with utDNA investigation in patients with MIBC, suggesting that a combination of ctDNA and utDNA might be a preferable strategy to detect MRD in these patients[112]. Lastly, in another study, sequencing of serial ctDNA samples collected following chemotherapy and radical cystectomy in patients with MIBC anticipated clinical metastases by 96 days in patients with metastatic relapse compared with cross-sectional imaging[95]. These results indicate that non-invasive plasma assays such as ctDNA can help detect disease recurrence substantially faster than traditional cross-sectional imaging. Importantly, whole-exome sequencing of a subset of the ctDNA samples collected at the time of relapse showed mutational concordance with the primary tumour, which might help in the selection of patients for systemic adjuvant or salvage therapy.

Additional supporting evidence for ctDNA as an MRD monitoring strategy to select patients for treatment came from a post hoc analysis of the IMvigor010 trial, in which treatment with atezolizumab was compared with observation in patients who had undergone surgical resection of high-risk, operable urothelial cancer[113]. The trial did not reach the efficacy end points of disease-free survival or OS in the intention-to-treat population, but ctDNA positivity at the beginning of adjuvant treatment was associated with a poor prognosis (HR 6.3, 95% CI 4.45–8.92; $P < 0.0001$)[114]. Furthermore, patients who were positive for ctDNA had improved disease-free survival (HR 0.58, 95% CI 0.41–0.96; $P = 0.0024$) and OS (HR 0.59, 95% CI 0.41–0.86) after being treated with atezolizumab compared with observation. Conversely, patients with negative ctDNA did not experience a similar survival benefit following treatment. Notably, even among patients with negative ctDNA, recurrence rates were still ~40%, suggesting that the genomic assays used might have had suboptimal sensitivity, leading to some false-negative results. These findings have crucial implications in the adjuvant systemic therapy setting, in which ctDNA can help identify patients at low risk of recurrence (ctDNA-negative), and in turn reduce over-treatment.

Considering the promising results of the IMvigor010 post hoc analysis, a clinical trial (IMvigor 011) was launched to assess the efficacy of adjuvant atezolizumab in 495 patients with ctDNA-positive, high-risk MIBC following radical cystectomy[115].

Additionally, another study (the treatment of metastatic bladder cancer at the time of biochemical relapse following radical cystectomy (TOMBOLA) trial) has been separately launched to investigate whether a ctDNA-guided treatment paradigm following surgery might improve outcomes in patients with MIBC[116]. In this study, the hypothesis that early treatment implementation upon detection of ctDNA can lead to superior oncological outcomes compared with conventional therapeutic delivery schedules will be assessed. The launch of both of these large-scale clinical trials shows that ctDNA status is a clinically relevant marker similar to stage, grade or detectable recurrences on imaging. This evidence constitutes a major paradigm shift in the adjuvant therapy landscape for advanced urothelial carcinoma, as in these patients treated for biochemically recurrent disease, ctDNA status will be used to drive therapeutic strategies.

Response monitoring

In urothelial and other cancers, ctDNA has been most extensively studied in the metastatic setting. ctDNA is detectable in the vast majority of patients with mUC, and the landscape of driver gene alterations of ctDNA reconstructs that in aggressive primary disease with similar patterns in the type and frequency of somatic alterations[26,97,117]. Deleterious alterations of *TP53*, *RB1*, *MDM2* and many chromatin modifiers were commonly found in ctDNA from patients with mUC[117]. APOBEC-related mutational signatures, which were described in TCGA for bladder cancer, have also been identified in ctDNA from patients with bladder cancer[97]. In a cohort of 104 patients with mUC undergoing systemic treatment, 83.4% of all mutations were found to be shared between the primary tumour tissue and ctDNA, with only 8.7% exclusively expressed by the tumour, indicating that ctDNA from peripheral plasma accurately captures the genomic profile of the primary tumour[97].

Levels of detected cfDNA can be used as a surrogate marker of disease burden, to evaluate treatment response and to discover mechanisms of acquired resistance. The adoption of liquid biopsies enables comprehensive evaluation of the systemic disease characteristics as well as serial sampling to accurately track the evolving mutational landscape of a tumour. The clinical utility of cfDNA has been shown in some clinical trials including patients with mUC, in which therapeutic efficacy was more closely associated with target alterations detected within cfDNA than in primary tumour samples[118].

Similar to the observations in other tumour types, suppression of detectable ctDNA has been associated with favourable clinical

outcomes in patients with locally advanced urothelial cancer and mUC[104]. In patients with MIBC undergoing neoadjuvant chemotherapy, detectable ctDNA before radical cystectomy predicted lack of pathological response[104]. On-treatment ctDNA levels also positively correlated with post-cystectomy disease progression, and the presence of ctDNA during the second cycle of chemotherapy predicted early recurrence with a sensitivity of 83% and a specificity of 100%[104]. Fluctuation of dominant clones and subclones within utDNA collected on-treatment provided evidence for tumour clonal evolution under therapy-induced selective pressure[104]. These findings were subsequently recapitulated in large cohorts of patients with MIBC treated with chemotherapy[67,95]. This evidence confirms that ctDNA and utDNA can be used to monitor response to therapy and identify potential therapy-induced changes to tumour genomics, which might further guide therapy selection.

Tracking ctDNA levels before and after the start of treatment might also be used to predict early response to therapy. In several studies, reductions in ctDNA levels following the initiation of systemic therapy have been shown, particularly in patients with mUC who experienced a clinical response[26,97]. Actionable mutations frequently found within ctDNA included alterations in *FGFR3*, *ERBB2*, *ERCC2* and *ARID1A*[26,97]. Patients with metastatic bladder cancer harbouring *ERCC* mutations in ctDNA were shown to have improved PFS following platinum chemotherapy[97], and patients with *ARID1A* mutations had an increased probability of responding to immune checkpoint blockade[26]; both results were consistent with evidence obtained in previous studies based on tumour samples[13,29,119]. Conversely, ctDNA TMB did not correlate with response to either platinum chemotherapy or immunotherapy[97]. Variability in genomic alterations observed in ctDNA samples serially collected during treatment was much smaller compared with differences observed in serial tumour biopsy samples, indicating that peripheral sampling of plasma for ctDNA during systemic therapy might reflect disease response with higher fidelity than repeated tissue sampling[97]. Notably, assessment of TMB in DNA from blood is substantially affected by the number of base pairs of DNA sequenced in the assay and the total amount of DNA extracted from the patient's sample[120]. However, new actionable mutations often emerge in samples collected during treatment, necessitating serial collections to inform treatment selection. The identification of new actionable mutations during systemic therapy suggests that ctDNA could capture tumour evolution during treatment, which might reflect tumour selection by systemic therapy, and drive the choice of subsequent therapeutic strategies based on genomic status.

In a study including patients with mUC, the predictive value of *FGFR3* alterations in ctDNA for response to infigratinib (pan-FGFR inhibitor) was assessed in patients with proven *FGFR3* alterations in primary tumour samples[121]. Overall, 68% of patients receiving treatment harboured *FGFR3* alterations in ctDNA. In a preliminary analysis of the study, a decrease in *FGFR3* mutations was observed during treatment, which seemed to correlate with increased duration of therapy and a reduction in tumour volume. Additionally, patients with a reduced number of somatic mutations had an increased probability of responding to treatment. These results indicate crucial differences in the genomic profiles of patients with urothelial carcinoma between those derived from UTUC and those derived from bladder cancer, with changes particularly detected in *FGFR3* fusions and mutations. Sequential ctDNA monitoring during therapy was also incorporated in a multi-arm adaptive clinical trial in which patients with advanced urothelial carcinoma received treatment with durvalumab (BISCAY trial)[114]. In this study, sequential increases in mutated *FGFR* in ctDNA of patients receiving durvalumab correlated with reduced radiographic response to therapy in cycle 2 and cycle 3, whereas high baseline somatic allele frequency in ctDNA was associated with reduced OS (HR 2.14, 95% CI 1.22–3.75)[114].

ctDNA monitoring in patients with urothelial carcinoma offers several advantages that might outperform current protocols, which heavily rely on cross-sectional imaging. ctDNA has been shown to accurately capture the genomic profile of the primary tumour, and also to have kinetics that correlate with disease progression and survival. For example, changes in ctDNA levels are observed before and after systemic therapy or surgery, and these treatments have the ability to suppress ctDNA, with a correlation that persists during therapy, after completion of therapy, and during surveillance. Lastly, ctDNA captures tumour evolution during surveillance and treatment, which will help identify new or predominant genomic alterations to enable physicians to change treatment strategies if necessary.

Challenges and limitations

Accurate determination of the mutational landscape, microsatellite instability and TMB in urothelial cancers requires the understanding of multiple important pitfalls and limitations. First, the assay interpretation must enable the carefully exclusion of genomic alterations associated with clonal haematopoiesis, as, especially in old patients, some mutations associated with a urothelial neoplasm can be erroneously ascribed to clonal haematopoiesis[96,97]. Second, the total amount of ctDNA extracted from a patient's blood samples greatly influences the sensitivity of the assay, and some alterations such as base substitution mutations can be detected much more easily in liquid biopsy samples than other mutations such as short insertions and deletions. Genomic rearrangements and fusions are also challenging to detect in liquid biopsy samples and, specifically, copy number changes (including both amplifications and homozygous deletions) are the most difficult mutations to detect[122]. Lastly, emerging evidence suggests that some systemic cytotoxic chemotherapies can increase blood TMB levels[68,123]. This effect of ctDNA on TMB led to the development of clinical trials in which drugs such as temozolomide are used to inactivate mismatch repair genes and, in turn, increase response to immune checkpoint inhibition therapy[124].

Conclusions

Results from existing studies provide substantial evidence to make a compelling case for integrating cfDNA into the management of urothelial cancer. Genomic alterations in patients with urothelial cancer can be assessed peripherally with robust reproducibility through blood and urine analysis with NGS, and can be leveraged for cancer diagnosis, treatment selection, response monitoring and disease surveillance. Plasma ctDNA could be used in combination with utDNA as a sensitive biomarker of extra-urothelial micrometastatic disease, both in the pre-surgical and post-surgical settings. In the metastatic setting, ctDNA might reflect disease burden, inform treatment selection, and help monitor disease progression and patient response to systemic therapy. The presence of ctDNA has been shown to anticipate clinical progression, and might supplant the use of cross-sectional imaging in the future.

The knowledge regarding urothelial tumour biology and treatment resistance is gradually growing, and granular data from cfDNA assays will increasingly be incorporated into clinical trial design. If high genomic alteration concordance rates between cfDNA and tumour samples are validated, liquid biopsy samples will undoubtedly become

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the preferred source of biomarkers to screen patients for enrolment in future clinical trials to assess various targeted therapeutic agents. Results from the IMvigor011 trial indicated that ctDNA might also be used to select patients with detectable MRD for enrolment in trials in which adjuvant therapies are assessed. The possibilities for improving the current management paradigm in urothelial cancer using liquid biopsies seem endless. With the maturation of the technology and the consequent increase in the sensitivity and robustness of cfDNA analysis, cfDNA could potentially drive future progress towards clinical personalized medicine.

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Author contributions

Competing interests

The authors declare no competing interests.

Additional information

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Peer review information *Nature Reviews Urology* thanks K. Junker and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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