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# Liquid biopsies for bladder cancer

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*Comment on:* Christensen E, Birkenkamp-Demtröder K, Nordentoft I, *et al.* Liquid Biopsy Analysis of FGFR3 and PIK3CA Hotspot Mutations for Disease Surveillance in Bladder Cancer. *Eur Urol* 2017;71:961-9.

**Abstract:** The development of accurate urinary biomarkers for the non-invasive detection of urothelial bladder cancer (UBC) could transform patient pathways by reducing reliance on cystoscopy, and the identification of highly prognostic (or even predictive) biomarkers could better guide patient management. A number of approaches are being utilised to address these challenges in both urinary- and plasma-borne tumour DNA (tDNA), so-called “liquid biopsies”. Next generation sequencing (NGS) and droplet digital PCR (ddPCR) allow detection of very low levels of such tDNA amongst a large excess of non-tumour DNA, the former permitting large mutation panels to be assessed and the latter potentially identifying ultrarare mutant alleles yet restricted for multiplexing. Christensen *et al.* recently published their data regarding a ddPCR approach for the detection of common *FGFR3* and *PIK3CA* mutations in urinary cell-free DNA (cfDNA) and circulating tumour DNA (ctDNA). In this proof-of-principle study, levels of mutant cfDNA in the urine of non-muscle-invasive bladder cancer (NMIBC) patients were shown to be positively correlated with tumour stage, grade and size, and a high initial level of mutant urinary cfDNA indicated future disease progression. In a cystectomy patient group, high mutant urinary cfDNA predicted future disease recurrence, the association being more pronounced with ctDNA. In this Perspective, we discuss these data in more detail and in parallel with the study’s limitations. We set these findings within the context of the field as a whole, highlighting important data from other groups, the strengths and weaknesses of alternative approaches, and the exciting and potentially significant future utilities of these techniques.

**Keywords:** Liquid biopsy; urinary biomarker; bladder cancer

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The development of accurate biomarkers for the non-invasive detection of urothelial bladder cancer (UBC) could transform patient pathways by reducing reliance on cystoscopy which is burdensome for patients and expensive for healthcare providers (1,2). In addition, the identification of highly prognostic (or even predictive) biomarkers could better guide patient management, and especially for those patients with high-risk non-muscle-invasive bladder cancer (HR-NMIBC) who represent a treatment challenge (3,4). However, despite decades of intensive research, the need for effective biomarkers for bladder

cancer detection and prognostication remains unmet (2,5). A small number of soluble urinary protein biomarkers (NMP22, BTA) or exfoliated cell tests based on proteins (ImmunoCyt) or aneuploidy (UroVysion) have obtained Food and Drug Administration (FDA) approval but have not been widely adopted due to limited sensitivity and/or specificity (5). More recently, research studies analysing the DNA in urinary cell pellets for changes in copy number, methylation status and somatic mutations have all shown potential for accurate non-invasive detection of bladder cancer (6-8). These tests still require thorough validation

**Table 1** The advantages and disadvantages of various approaches to the analysis of urinary tDNA

Method	Disadvantages	Advantages
DNA methylation	Relatively large amount of DNA required Influenced by other factors (age, smoking)	Very high specificity and sensitivity recently reported (7)
Copy number changes	Unable to detect low levels of tumour DNA in a high background of non-tumour DNA	High specificity and most UBCs have copy number changes (10)
Microsatellite analysis	Unable to detect low levels of tumour DNA in a high background of non-tumour DNA Multiple individual tests needed	Good sensitivity and specificity reported (11)
Mutations	Multiple mutations must be analysed to achieve high sensitivity	High specificity and can detect low levels of tumour DNA in a high background of non-tumour DNA (8,12,13)

tDNA, tumour DNA; UBCs, urothelial bladder cancers.

both in the incident and recurrent disease settings. Pitfalls include obtaining sufficient high-quality DNA from all urine samples, dilution of tumour DNA (tDNA) with non-tumour DNA, the heterogeneity of bladder cancer and, for surveillance, verifying biomarkers that are absolutely cancer specific, i.e., are not seen in any “field effect” (9). The advantages and disadvantages of these approaches are summarised in *Table 1*.

Disease heterogeneity requires that either multiple markers need to be measured for a broadly applicable test which detects as many tumours as possible (high sensitivity), or the biomarker test needs to be individualised to the characteristics of a patient’s disease. The latter approach relies on the hypothesis that recurrent disease will retain the same genomic aberrations as the primary tumour (9,14). The former approach may require measuring a combination of alterations in copy number, methylation, and mutations at very low mutant allele frequencies; further technological innovation is needed to allow these diverse changes to be detected using a single analytical platform.

Two recent technologies that allow detection of very low levels of tDNA amongst a large excess of non-tumour DNA are droplet digital PCR (ddPCR) and next generation sequencing (NGS). ddPCR is ideal for detecting ultra-rare alleles and gives absolute quantitation of the number of mutant alleles, but is limited in its ability to multiplex, i.e., large numbers of biomarkers cannot be assayed simultaneously. ddPCR is therefore ideal for developing individualized biomarker assays. Conversely, NGS is able to measure a large number of biomarkers (up to whole genome level), enabling the development of broad applicability tests, but is limited in its ability to detect very

low mutant allele frequencies. With current technology and using high read depths, the lower limit of detection of NGS is determined by the background noise and is of the order of 1% mutant allele frequency. NGS workflows that incorporate unique molecular identifiers (so that reads can be traced to original DNA molecules in the specimen) enable detection of one tDNA molecule amongst >1,000 wtDNA molecules (15); however, these approaches may be difficult to apply for multiple markers, especially when low nanogram quantities of DNA are available (as often occurs with urine), and require ultra-high read depths, thus increasing expense. Targeted NGS has been successfully used for the detection of mutations in *FGFR3* and *TERT* individually, and as part of a 6-gene panel in urinary cell-pellet DNA (8,13,16). It should be noted that low DNA yields from some urine specimens will provide only a few hundred/low thousand genome equivalents.

Whatever analytical approach is used, it is essential that the biospecimen contains sufficient tDNA to be detected, and urine DNA can be considered as two types which are easily separated by centrifugation or filtration: genomic DNA in exfoliated cells (the cell pellet) and cell-free DNA (cfDNA) in the supernatant/filtrate. We have observed that urinary cfDNA consists of slightly larger fragments than plasma cfDNA with a broad size distribution peaking between 200 and 300 bp, and a median concentration of 4 ng/μL in UBC patients (unpublished data). Two studies have directly compared the utility of urinary cell pellet DNA and urinary cfDNA for detecting bladder cancer using microsatellite analysis or copy number changes (6,17). Both found a higher proportion of tDNA relative to non-tumour DNA in the supernatant than in the cell pellet. This raises

the fascinating proposition that the various biomarker assays that have previously worked well on cell pellet DNA might work even better on supernatant cfDNA. Furthermore, the size-based capture and enrichment of tumour cells may also improve the identification and analysis of urinary tDNA (18), and the presence of circulating tumour cells have prognostic value (19).

In 2016, Birkenkamp-Demtröder *et al.* used NGS to detect chromosome breakpoints in the bladder tumours of 12 patients (20). They subsequently designed ddPCR assays targeting these variants and assayed cfDNA isolated from longitudinally collected urine and plasma samples. The somatic variants were detectable in both the plasma and urine of patients, even in non-muscle-invasive bladder cancer (NMIBC) patients. The levels of tDNA dropped in patients post-treatment and high levels were predictive of progression.

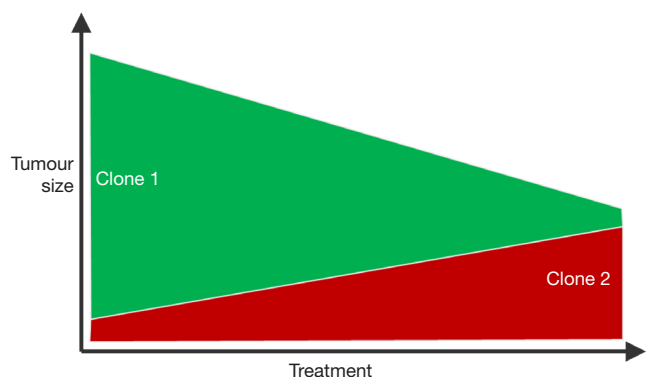
The same group have now expanded on the urine and plasma cfDNA work with a ddPCR study of common *FGFR3* and *PIK3CA* mutations (21). Theoretically, these markers should be applicable for a large proportion of bladder cancer patients (especially those with low-grade NMIBC), although additional “personalised” markers based on tumour sequencing would have to be used for the remainder of patients [or, alternatively, a broader panel (8)]. Initially, Christensen *et al.* tested over 800 formalin-fixed paraffin-embedded (FFPE) tumour tissues for the S249C and Y373C *FGFR3* and E545K *PIK3CA* mutations, with 172 tumours testing positive (36% of NMIBCs and 11% of patients undergoing cystectomy); 54 of these patients were selected for subsequent urine and plasma cfDNA analysis, with selection based upon the availability of “liquid biopsies” and disease course characteristics.

ddPCR data provide an absolute determination of the number of copies of mutant DNA per volume of body fluid. The absolute levels of mutant cfDNA in the urine of NMIBC patients were shown to be positively correlated with tumour stage, grade and size, although the association with European Organisation for Research and Treatment of Cancer (EORTC) risk category did not reach statistical significance (22). In the NMIBC patient group (n=25), tDNA was not detected in plasma but a high initial level of urinary supernatant tDNA indicated future disease progression (P=0.036). In the cystectomy patient group (n=27), high urinary supernatant tDNA also appears to be a predictor of future recurrence (P=0.031); this effect is more pronounced with plasma tDNA/ctDNA (P<0.001).

This is interesting research, although a number of

aspects require clarification and further study. The small number of patients whose liquid biopsies were ultimately analysed were enrolled from 1992 to 2012, and so there is understandably a spectrum of treatment approaches over time. Intravesical Bacillus Calmette-Guérin (BCG) appears to have been utilised sparingly in those patients with T1 NMIBC, and cystectomy appears to have been applied inconsistently to those NMIBC patients who progressed to MIBC (3); furthermore, no patient undergoing cystectomy received neoadjuvant chemotherapy (23). All of these treatment factors would have a significant influence on outcomes and, potentially, the persistence or re-emergence of tDNA, or the ability for mutations to be detected by the assay. And despite the urgent need for better prognostic biomarkers for HR-NMIBC patients, very few initial stage T1 patients were analysed (n=5). Furthermore, as the authors highlight, urinary supernatant tDNA may originate from renal clearance of tDNA in the circulation, and so the presence of the E545K *PIK3CA* mutation may not be bladder cancer specific (24).

In addition, the concept of “anticipatory” or “pre-emptive” diagnosis is a well-known pitfall in the field of non-invasive UBC diagnosis, whereby test specificity can be adversely affected by either the “field effect” and persistence of tumour-related abnormalities in the urine after clearance of disease (9), or by recurrent or residual disease that is not visible by white light cystoscopy (25-29). These phenomena also have implications for prognostication. In this study the majority of NMIBC patients whose samples were selected for plasma and urinary tDNA analysis experienced recurrence or progression [Figure 3 in the paper (21)]: only 2 of the 25 (8%) patients experienced sustained event-free survival at a median follow-up of 59 months, and neither had persistence of plasma or urinary tDNA after the tumour episode. By not studying more patients with such a prolonged event-free survival, the authors have perhaps missed an opportunity to demonstrate the frequency of urinary tDNA persistence in patients whose bladders have seemingly been successfully treated. The selected NMIBC patients also experienced a very high rate of residual disease: almost two-thirds of T1 episodes were seemingly followed by recurrence or progression at the next cystoscopy episode after treatment. Therefore, although the data show that the presence of urinary tDNA in NMIBC patients is significantly associated with progression of disease, it is equally likely to be indicative of inadequate treatment by resection and/or intravesical therapy. A contemporary cohort where patients receive current guideline-directed



**Figure 1** Illustration of how a therapy may eradicate the predominant clone within a tumour (clone 1, green), accompanied by tumour shrinkage, yet in parallel a potentially more aggressive treatment-resistant clone thrives (clone 2, red). It is feasible that genome-wide or panel-based approaches to the analysis of urinary or plasma tDNA during treatment may demonstrate such phenomena and could be performed regularly, whereas one would not invasively biopsy a bladder tumour on a regular basis to identify the same phenomena. A liquid biopsy may also capture disease heterogeneity better than a solid biopsy which only samples a single part of a single tumour. Clearly, *in vivo*, the scenario is far more complex with multiple clones within the same tumour vying for survival, and so this illustration is a gross oversimplification.

treatment may reveal a lower frequency of urinary tDNA post-treatment and during surveillance, but the relationship with subsequent progression may be more robust. And a similar phenomenon might be observed in patients treated with neoadjuvant chemotherapy prior to cystectomy.

In the future, utilising genome-wide or panel-based approaches for the analysis of plasma or urine tDNA may permit the near real-time monitoring of tumour evolution during intravesical therapy, neoadjuvant chemotherapy or chemoradiotherapy (6,30), possibly permitting adjustments to therapeutic approaches (*Figure 1*).

However, despite its limitations, the authors of this study are to be commended on advancing the non-invasive characterisation of bladder cancer. Whilst it is a proof-of-principle study analysing a small number of patients and their liquid biopsies, it clearly points to a bright future for cfDNA-based tests in improving the management of bladder cancer patients (6). The bladder cancer community should now focus on selecting the best biomarkers, optimising assay sensitivity, and large-scale validation of these novel tests.

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## Footnote

*Conflicts of Interest:* RT Bryan has contributed to advisory boards and has received honoraria for teaching from Olympus Medical Systems on the topic of narrow band imaging (NBI) cystoscopy; the other author has no conflicts of interest to declare.

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