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Platinum Priority – Bladder Cancer
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Genomic Alterations in Liquid Biopsies from Patients with Bladder Cancer

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Abstract

Background: At least half of the patients diagnosed with non–muscle-invasive bladder cancer (NMIBC) experience recurrence and approximately 15% will develop progression to muscle invasive or metastatic disease. Biomarkers for disease surveillance are urgently needed.
Objective: Development of assays for surveillance using genomic variants in cell-free tumour DNA from plasma and urine.
Design, setting, and participants: Retrospective pilot study of 377 samples from 12 patients with recurrent or progressive/metastatic disease. Three next-generation sequencing methods were applied and somatic variants in DNA from tumour, plasma, and urine were subsequently monitored by personalised assays using droplet digital polymerase chain reaction (ddPCR). Samples were collected from 1994 to 2015, with up to 20 yr of follow-up.
Outcome measurements and statistical analysis: Progression to muscle-invasive or metastatic bladder cancer; *t* test for ddPCR data.
Results and limitations: We developed from one to six personalised assays per patient. Patients with progressive disease showed significantly higher levels of tumour DNA in plasma and urine before disease progression, compared with patients with recurrent disease ($p = 0.032$ and 1.3×10^{-6}, respectively). Interestingly, tumour DNA was detected in plasma and urine in patients with noninvasive disease, being no longer detectable in disease-free patients. A significant level of heterogeneity was observed for each patient; this could be due to tumour heterogeneity or assay performance.
Conclusions: Cell-free tumour DNA can be detected in plasma and urine, even in patients with noninvasive disease, with high levels of tumour DNA detectable before progression, especially in urine samples. Personalised assays of genomic variants may be useful for disease monitoring.
Patient summary: Tumour DNA can be detected in blood and urine in early and advanced stages of bladder cancer. Measurement of these highly tumour-specific biomarkers may represent a novel diagnostic tool to indicate the presence of residual disease or to discover aggressive forms of bladder cancer early in the disease course.

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1. Introduction

Bladder cancer (BCa) is the fifth most common cancer in the Western world [1]. About 75% of patients with BCa are diagnosed with non–muscle-invasive BCa (NMIBC) and at least half of the patients have a high recurrence and moderate progression rate (approximately 15%) to muscle-invasive BCa within 5 yr [1,2]. Major clinical challenges are to identify patients at risk for recurrence, progression, and metastasis, and to monitor treatment response. Promising biomarkers have been identified in genomic [3], epigenetic [4], transcriptomic [5,6], and proteomic studies [7]. Furthermore, several studies have focused on delineating diagnostic biomarkers for BCa surveillance [8]. None of the biomarkers have yet reached the clinic because of limited validation and relatively low sensitivity and specificity.

The mutational architecture of bladder tumours has been described by several groups [9–16]. In a recent study, we identified genomic variants in low-stage as well as advanced, localised tumours and found that metachronous tumours shared 25–56% of high-impact mutations and structural rearrangements, thus indicating that genomic changes may be used for BCa surveillance [15]. DNA is continuously released into the blood circulation. In cancer patients, the majority of plasma DNA originates from healthy cells, whereas a minor fraction is tumour DNA [17]. The circulating tumour DNA (ctDNA) contains tumour-specific genomic variants that may be used as unique genetic signatures or biomarkers [18]. Novel techniques identified these genomic variants in DNA from plasma [19]. Genomic variants in cell-free tumour DNA were detected in 75% of patients with advanced cancers in one study [20]. Furthermore, monitoring of large chromosomal rearrangements in plasma from colorectal or breast cancer patients provided evidence that a molecular relapse by ctDNA can be detected earlier than a clinical relapse based on image diagnostics [21].

Here, we established methods for noninvasive disease surveillance in patients with NMIBC, using highly sensitive and tumour-specific personalised plasma- and urine-based assays. Using next-generation sequencing, we identified somatic genomic variants in tumour DNA from six patients with recurrent disease and six with progressive and metastatic disease. Although this is a retrospective study with a relatively low number of patients, we demonstrate that surveillance of BCa patients using plasma- or urine-based personalised assays may be a promising approach for detection of progression and metastatic disease. Importantly, we also showed that ctDNA levels were low, but readily detectable, in plasma from patients without disease progression, whereas they disappeared in disease-free patients.

2. Patients and methods

2.1. Patients and clinical samples

In total, 377 samples comprising blood, tumours, plasma, and urine from six progressive patients (PRO group) and six recurrent patients (REC group) diagnosed with NMIBC were collected between 1994 and 2015 at Aarhus University Hospital, Aarhus, Denmark (Table 1). Patients were followed according to national guidelines and detailed follow-up data were available for all patients. Patients were selected from our biobank based on various criteria (Supplement 1). All patients provided written informed consent, and the study was approved by The National Committee on Health Research Ethics.

2.2. Biospecimen collection and DNA extraction

Fresh tissue samples were stored at −80 °C. Median carcinoma cell percentage was >80% (Table 1). EDTA (Ethylenediaminetetraacetic acid) blood was collected at each visit and centrifuged at 3000 *g* for 10 min. Plasma was centrifuged at 3000 *g* for 5 min. DNA was extracted from an average of 2.2 ml plasma or 3.4 ml urine (Table 2), using the QIAsymphony Circulating NA kit (Qiagen, Hilden, Germany) (Supplement 1).

2.3. Sequencing and variant calling

Three methods were applied to identify genomic variants in tumour and matching germline DNA: whole genome sequencing, whole exome sequencing, and mate-pair sequencing (Supplement 1; Supplementary Fig. 1; Supplementary Table 1a–1c).

2.4. Polymerase chain reaction validation of genomic variants

Genomic variants were evaluated by polymerase chain reaction (PCR) analysing tumour and matched germline DNA (Supplementary Table 1a–1c), using amplicons of 68–300 base pairs (Supplement 1).

2.5. Development of personalised assays

We prioritised genomic variants in common between at least two metachronous tumours (methods 1 and 2), supported by most read pairs or affecting loci harbouring Catalogue of Somatic Mutations in Cancer (COSMIC) cancer genes [22] (method 3). We validated 4–48 genomic variants by PCR, using tumour and matched germline DNA, and the precise breakpoint at base-pair resolution was determined by Sanger sequencing (Supplementary Fig. 1). Breaks were considered tumour specific if absent in matched germline DNA (Supplementary Table 2).

3. Results

We identified tumour-specific genomic variants to develop specific personalised assays for disease surveillance, using liquid biopsy specimens. A schematic overview is shown in Figure 1.

3.1. Defining genetic signatures for individual tumours

We sequenced 22 tumours and matched germline DNA from 12 patients initially diagnosed with NMIBC (Table 1). The PRO group ($n = 6$) included patients with later progression to muscle-invasive or metastatic disease followed for 4–20 yr. The REC group ($n = 6$) included patients with recurrence of NMIBC followed for 7–20 yr, all of whom were alive at the time of analysis (Table 1). We identified varying numbers of tumour-specific

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Table 1 – Clinical and histopathologic data from 12 patients initially diagnosed with non–muscle-invasive bladder cancer

Patient ID	Sex*	Follow-up†, mo	Age, yr	Visit, no.	Tumour stage ‡	Tumour grade ‡	Size, cm	Carcinoma cells, % §	Resection, ‖, mo	Method no.**	Treatment no.††	M.S. ‡‡	End point §§
PRO group													
01	F	60	70	1	Ta	LG	<3	95	1	1			
			73	7	T2	HG	≥3	90	33	1	1,3	–	D
04	M	103	67	1	Ta	HG	<3	95	1	1			
			68	4	T2	HG	≥3	95	22	1	4,2	1	DF
05	M	237	63	2	Ta	LG	<3	90	9	2			
			79	33	Ta	HG	<3	95	211	2,3			
			80	35	Ta	HG	<3	45	219	n.a.			
			80	36	T2	HG	<3	n.a.	223	n.a.	–	2,3	D
07	M	47	65	1	Ta	LG	<3	n.a.	1	n.a.			
			68	12	T2	HG	≥3	60	45	3	1,2	2,3,4	D
10	M	96	79	1	Ta	LG	<3	50	1	n.a.			
			85	4	T1	LG	<3	60	76	n.a.			
			86	6	T1	LG	<3	65	85	3			
			89	7	T4	HG	<3	n.a.	86	n.a.	1,3	4	D
14	M	48	85	1	Ta	LG	≥3	90	1	n.a.			
			85	4	Ta	LG	≥3	85	8	n.a.			
			88	12	T2	HG	<3	50	45	3	1	5	D
REC group													
02	M	124	67	1	Ta	LG	<3	90	1	1			
			68	2	Ta	LG	<3	95	9	n.a.			
			68	3	T1	HG	<3	95	18	1	1	–	DF
03	F	113	74	1	Ta	LG	<3	90	1	1			
			75	3	T1	HG	<3	95	33	1			
			75	5	Ta	HG	<3	80	68	n.a.	1	–	DF
06	M	237	44	2	Ta	LG	<3	90	1	2,3			
			47	8	Ta	LG	<3	80	37	2			
			49	12	Ta	LG	<3	80	67	2			
			52	14	Ta	LG	≥3	80	97	2			
			52	15	Ta	LG	<3	95	101	2	–	–	DF
11	M	91	75	1	Ta	LG	<3	80	1	2,3			
			78	8	Ta	HG	<3	80	33	2	–	–	DF
12	F	89	73	1	Ta	LG	<3	100	1	3			
			75	5	Ta	LG	<3	95	23	n.a.	–	–	DF
13	M	87	64	2	Ta	LG	<3	80	4	3			
			65	7	Ta	LG	<3	95	23	n.a.	1	–	DF

D = death from disease; DF = disease free; F = female; HG = high grade; LG = low grade; M = male; M.S. = metastatic site; n.a. = not applicable; NMIBC = non–muscle-invasive bladder cancer; PRO = progression; REC = recurrence.

* All patients were current or former smokers except patient 12.

† Total follow-up time was defined as the time from the first stage Ta tumour sample (visit 1) until the latest recorded visit or until death from disease. Five of six patients in the PRO group were already diagnosed with NMIBC before our sampling started; this period was not taken into account in Table 1. The recorded time with bladder cancer before sampling is indicated in Figure 2. Average total follow-up time was 98.5 mo for the PRO group and 123.5 mo for the REC group.

‡ The World Health Organization system of malignancy grading was used.

§ The mean carcinoma cell percentage was 82%.

‖ Time at resection of the indicated tumour by transurethral resection of the bladder; the first sampling (visit 1) corresponds to the first month. The period with NMIBC before sampling started was not taken into account.

** Sequencing and analysis method applied to these samples (workflow methods 1–3 in Supplementary Fig. 1).

†† Treatment: 1 = bacillus Calmette-Guérin; 2 = chemotherapy; 3 = irradiation; 4 = cystectomy.

‡‡ 1 = lymph node; 2 = lung; 3 = liver; 4 = bones; 5 = cerebral (suspect).

§§ Patients with end point DF were still alive at the latest recorded visit 87–237 mo after diagnosis of NMIBC with follow-up still ongoing.

genomic variants (Supplementary Table 1a–1c). Characteristics of the three methods applied are described in Supplement 1.

3.2. *Developing clinically applicable assays for the genetic signatures*

We designed one to six tumour-specific personalised assays (46 total) (Supplement 1; Supplementary Table 2). The assays had an average linearity of $R^2 = 0.9988$ and were highly sensitive, detecting 1 copy in 6000 background copies (Supplementary Fig. 2).

3.3. *Comparison between metachronous tumours*

To validate assay performance and intrapatient heterogeneity, we initially analysed the tumours (Table 1) and quantified the level of variant DNA within the total tumour DNA (Supplementary Fig. 3). All assays were 100% tumour specific, as no signal was detected in matched germline DNA, and all assays confirmed the patient-specific variants in the tumour used for sequencing. These variants were also identified in tumours from additional visits. We observed a slightly larger variation in samples from the PRO group, indicating larger intertumour heterogeneity and suggesting

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Table 2 – Analysis of cell-free tumour DNA from plasma and urine

	Plasma		Urine	
	cfDNA	ctDNA	ucfDNA	Tumour ucfDNA
REC group	Patients* 02, 03, 11, 12, 13		Patients 02, 03, 06, 11, 12, 13	
Mean volume, ml (range)	2.2 (0.8–4.0)		3.4 (2–4.5)	
Analyses, no.	44	146	44	126
Mean no. copies/ml (range)	2867 (404–9118)	11 (0–120)	2018 (0–36500)	31 (0–812)
PRO group	Patients * 01, 04, 07, 14		Patients 01, 04, 05, 07, 10, 14	
Mean volume, ml (range)	2.3 (1.5–4.0)		3.1 (0.8–4.5)	
Analyses, no.	22	113	48	189
Mean no. copies/ml (range)	3049 (838–9710)	138 (0–6384)	37 256 (0–654872)	1242 (0–25194)
t test, *p* value†	7.3×10^{-1}	3.2×10^{-2}	5.8×10^{-2}	1.3×10^{-6}

cfDNA = cell-free DNA; ctDNA = circulating tumour DNA; PRO = progression; REC = recurrence; ucfDNA = cell-free DNA in urine.
* Patient 05 (PRO) with tumour DNA detected in only one plasma sample and patient 10 (PRO) and patient 06 (REC) with no tumour DNA detectable in the volume of plasma used here were excluded.
† Comparison of the PRO group and the REC group (exclusively samples obtained before diagnosis of clinical progression). Samples obtained at or after clinical progression to muscle-invasive or metastatic bladder cancer were excluded from analysis.

the use of several assays per patient to increase the probability of detecting tumour DNA during surveillance.

3.4. *Noninvasive monitoring of clinical outcomes: plasma analysis*

We analysed DNA from 115 plasma samples obtained at several visits (Table 2; Supplementary Table 3). No significant difference was observed for total circulating DNA between the PRO and REC groups (*t* test, $p = 0.104$). Applying personalised assays, tumour DNA was detectable in plasma in 83% (10 of 12) of the patients. In 67% (four of six) of the PRO-group patients, tumour DNA was detected several months before clinical progression (Fig. 2; Supplementary Fig. 4 and 5). No tumour DNA was detectable in plasma from two patients (REC-group patient 06 and

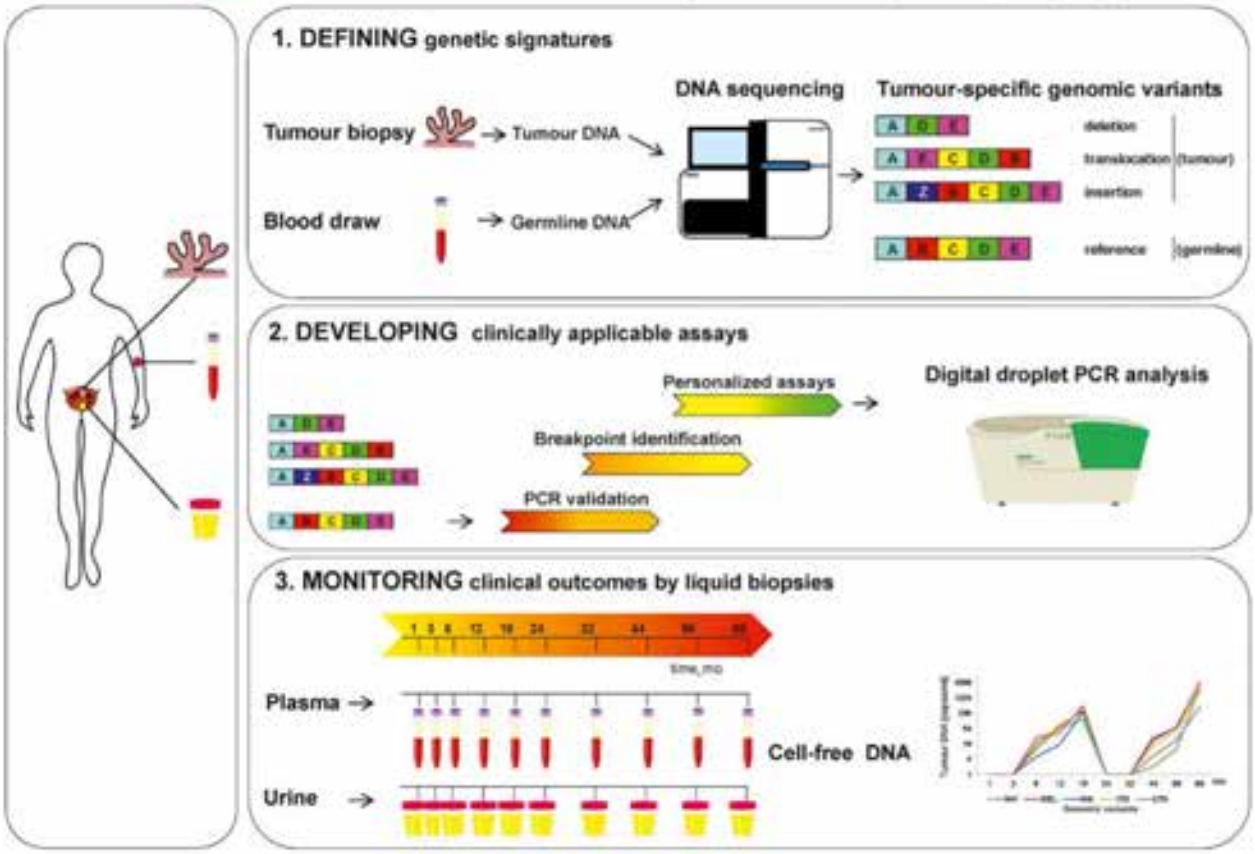

Fig. 1 – Schematic overview of the personalised assay design strategy. Tumour DNA and matched germline DNA were sequenced and tumour-specific genetic signatures (somatic genomic structural variants) were identified by bioinformatic approaches. Genomic variants were validated by polymerase chain reaction (PCR) and, if necessary, the precise breakpoints were determined by Sanger sequencing. Tumour-specific, highly sensitive, personalised assays spanning the breakpoints were designed and used to analyse liquid biopsy specimens (plasma and urine), consecutively sampled over several years, by droplet digital PCR.
CTX = interchromosomal translocations; DEL = deletions; INS = insertions; INV = inversions; ITX = intrachromosomal translocations; PCR = polymerase chain reaction.

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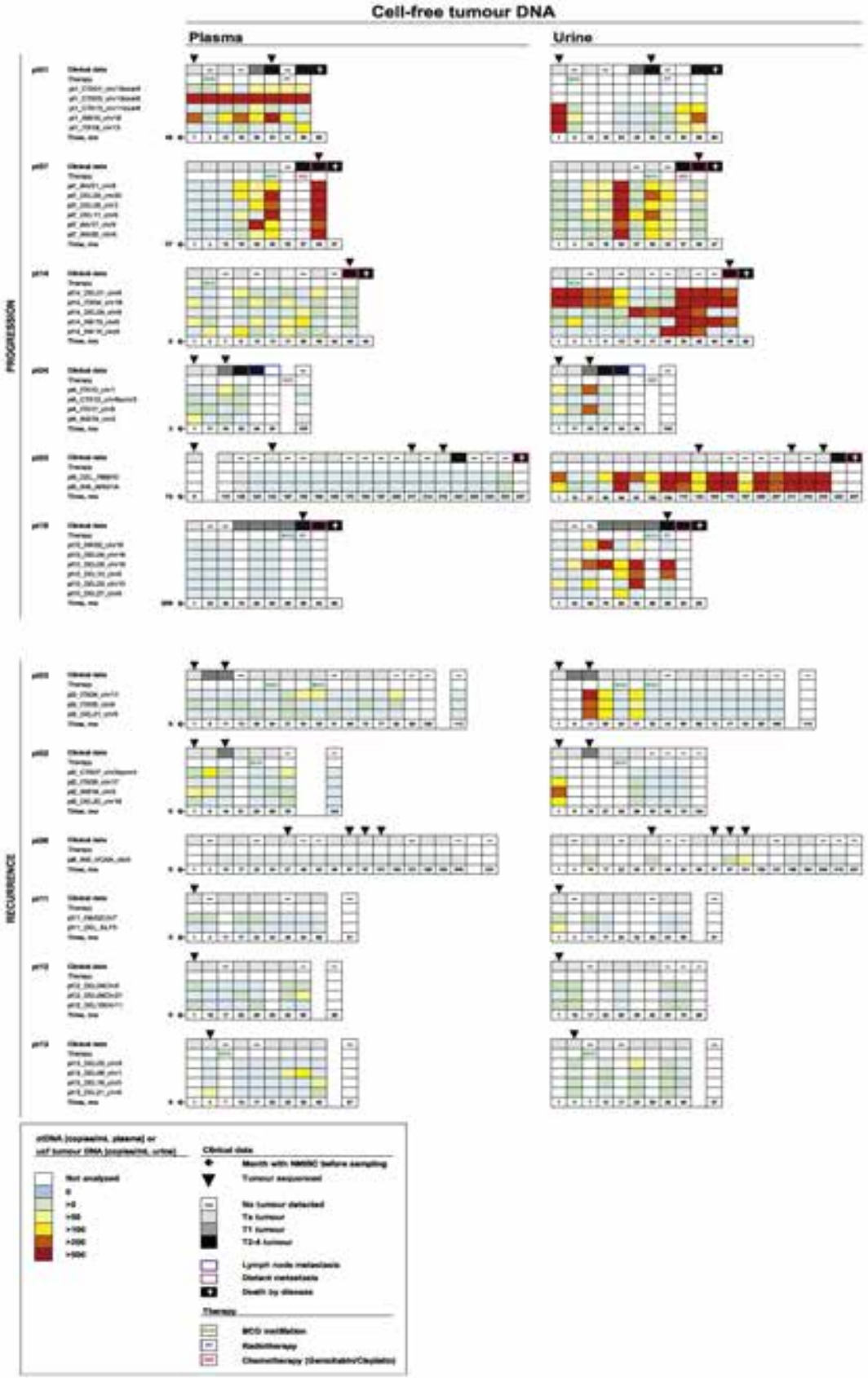

Fig. 2 – Analysis of cell-free DNA from plasma and urine. Quantification of genomic variants from patients with progressive (patients 01, 07, 04, 05, 10) or recurrent (patients 03, 02, 06, 11, 12, 13) NMIBC in cell-free tumour DNA from plasma (left) and urine (right). For each patient, tumour stage and therapy are indicated. Personalised assays assessing copies per millilitre of somatic genomic variants in consecutive samples obtained over time are shown by a colour code.
BCG = bacillus Calmette-Guérin; NMIBC = non–muscle-invasive bladder cancer; pt = patient.

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PRO-group patient 10). The average tumour DNA level was significantly higher in the PRO group when analysing samples from all visits during the disease course (average 154 vs 11 copies/ml, respectively; *t* test, $p = 5.4 \times 10^{-4}$) (Supplementary Table 3). This was also observed for samples exclusively obtained before diagnosis of progression to muscle-invasive disease (average 138 vs 11 copies/ml; *t* test, $p = 0.032$) (Table 2).

For the PRO group, we observed high levels of tumour DNA in the plasma of patient 01 throughout the disease course and an increase of DNA over time in patient 07. For patient 14, tumour DNA was detectable in plasma from all visits, showing different types of variants released into circulation. Remarkably, no tumour DNA was detectable 6.5 yr after surgery in patient 04, who experienced relapse after cystectomy followed by complete remission after systemic therapy (Supplementary Fig. 4). Importantly, no or very low levels of tumour DNA (<60 copies/ml) were detected for the five patients from the REC group with detectable tumour DNA at previous visits. Supplementary Table 4 gives a detailed overview of the type and number of samples analysed for each patient.

3.5. *Noninvasive monitoring of clinical outcomes: urinary analysis*

Cell-free urine contains highly fragmented DNA derived from urothelial cells, inflammatory cells, or transrenal DNA fragments cleared from circulation [23]. We analysed 101 of 116 urine samples, using personalised assays (Supplementary Table 3). We detected tumour DNA in 96.5% (55 of 57) of the urine samples from the PRO-group patients, but in only 50% (22 of 44) of those from the REC group (Table 2; Fig. 2; Supplementary Fig. 6 and 7). Importantly, all PRO-group patients showed high levels of tumour DNA; in five of six patients, including those with very low or no detectable tumour DNA in plasma, >500 copies/ml were present in urine. Patients 02 and 03 in the REC group had high levels of tumour DNA in their urine early in the disease course, probably originating from the patients' T1 tumours (Fig. 2; Supplementary Fig. 7). The average tumour DNA levels were significantly higher in the PRO-group patients than in the REC-group patients, when analysing all urine samples during the disease course (average 1282 copies/ml vs 31 copies/ml, respectively; *t* test, $p = 0.007$) (Supplementary Table 3). Furthermore, we found a highly significant difference in the tumour DNA level in urine samples obtained at visits before diagnosis of progression to muscle-invasive disease exclusively (average 1242 copies/ml vs 31 copies/ml; *t* test, $p = 1.3 \times 10^{-6}$) (Table 2). Overall, high levels of tumour DNA were found in urine samples of 83% of patients from the PRO group several months before clinical progression to muscle-invasive disease (12–169 mo). In conclusion, the analysis of genomic variants in DNA from urine provides an efficient diagnostic tool for noninvasive, frequent monitoring of patients with BCa.

Tumour-specific genomic variants were detected in DNA from urine pellets without a significant difference between the groups ($p = 0.763$) (data not shown).

4. Discussion

The use of next-generation sequencing has fuelled individualised medicine. In this retrospective pilot study, we developed tumour-specific, personalised assays for 12 patients with BCa and analysed DNA from 377 samples taken throughout the patients' disease courses. Significantly higher levels of tumour DNA in liquid biopsy specimens were detected before disease progression occurred. High levels of tumour DNA were detected in urine samples from all patients with progressive disease, including those without detectable tumour DNA in the plasma, whereas only low levels were detected throughout the disease course of patients with recurrent disease.

For patient 07 in the PRO group, tumour DNA was already detectable about 1 yr before progression and increased fivefold over time. High tumour DNA levels (>2000 copies/ml plasma) were detected 8 mo after systemic therapy, correlating with relapse and disease progression. No tumour DNA was detectable in the plasma from two patients. Patient 06 in the REC group, who had recurrent stage Ta tumours, was followed for 20 yr and was disease free for 2.5 yr. We assume that the plasma may have contained only a few copies of tumour DNA and so it was below the assay detection limit. No tumour DNA was detectable in the plasma from patient 10 in the PRO group, despite the use of six personalised assays. Analysis of consecutive tumour biopsies from patient 10 showed a large intertumour heterogeneity, with only 1 of 48 genomic variants found to be of clonal origin. It is uncertain whether bone metastases were derived from the urothelial tumour in the bladder or a muscle-invasive urothelial tumour evolved in the prostatic urethra with a potentially different genomic profile. It has been shown that the level of variants detectable in plasma reflects the clonal hierarchy [24]. We cannot exclude that other genomic variants than those analysed may have been released into circulation, due to a shift in cell populations. In a recent study, no tumour DNA was detectable in about 10% of patients with metastatic colorectal cancer [25], suggesting that some patients may not release tumour DNA into circulation, or they may have a high renal clearance.

Interestingly, tumour DNA was detected in plasma from patients with noninvasive disease. This finding was striking, as Ta tumours should have an intact basal membrane. It has been suggested that the tumour interstitial fluid may play a role as a possible source of biomarkers [26] in which exosomes may support the transcapillary transport of DNA into the circulation [27].

In urine, high levels of tumour DNA were detected in all patients with progressive disease, compared with low levels in samples from patients with recurrent disease. We observed significantly higher levels of tumour DNA in urine from patients with invasive/metastatic disease than in patients with recurrent, superficial tumours, indicating that the levels of cell-free tumour DNA are reflecting the invasiveness rather than simply tumours present in the bladder. Interestingly, patients 02 and 03 in the REC group had high levels of tumour DNA associated with diagnosis of high-risk T1 tumours, but after bacillus Calmette-Guérin

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instillation, no tumour DNA was detectable, suggesting a potential application to monitor response. Remarkably, in patient 07, the tumour DNA in urine sampled at the initial visit already contained the five genomic variants identified in the muscle-invasive tumour resected 4.5 yr later, and patient 14 in the PRO group had high levels of tumour DNA already at the diagnosis of NMIBC, with increasing levels over time. For both patients, routine urine analyses may have been a good supplement to conventional cystoscopy.

Although the number of patients was limited, this is the first study to suggest that the level of tumour DNA in plasma and urine may be a useful tool for disease surveillance. Analyses of larger cohorts of patients are needed to define a statistical significant molecular threshold to discriminate high- and low-risk patients. Our study provides evidence that cell-free tumour DNA is already detectable in plasma and urine at the diagnosis of NMIBC, as well as in consecutive samples obtained at several visits over time.

The personalised assays may provide clinicians with a measure of disease aggressiveness and early signs of progression and invasion. For clinical application, patients could be classified by progression markers, for example [6]. Genomic variants could be identified in a tumour biopsy specimen from high-risk patients, followed by design of personalised assays and detection of tumour DNA in liquid biopsy samples. Another strategy may be to apply predesigned assays for detecting activating mutations in *FGFR3*, *TERT*, or *PIK3CA*. Targeted sequencing of circulating tumour DNA identified mutations from metastatic colon and breast cancer patients [28]. This method allows tracking of tumour evolution over time [29,30], thereby eliminating erroneous analysis of variants absent in circulation. In the future, a potential application might be monitoring response to chemotherapy to provide clues about the clonal population developing over time and thus monitoring therapeutic response [25] or offering the possibility to customise systemic therapy approaches [31]. Additional studies are needed to address whether targeted sequencing is applicable to patients diagnosed with NMIBC, or whether the targeted approach should be restricted to advanced disease, where higher levels of tumour DNA are expected.

5. Conclusions

The present study points to several clinical perspectives for the future use of personalised genomic biomarkers. Early detection of progression and identification of disease-free patients may improve disease surveillance and patient outcome. Liquid biopsy specimens are under investigation in several trials [19]. However, affordable and time-saving methods are needed and the use of liquid biopsy biomarkers have to be validated in prospective and sufficiently powered multicentre studies to determine feasibility and thresholds of cell-free DNA levels for discriminating between low- and high-risk patients.

Author contributions: Karin Birkenkamp-Demtröder had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Birkenkamp-Demtröder, Dyrskjøt.
Acquisition of data: Birkenkamp-Demtröder, Nordentoft, Vang.
Analysis and interpretation of data: Birkenkamp-Demtröder, Dyrskjøt, Nordentoft, Reinert, Ørntoft.
Drafting of the manuscript: Birkenkamp-Demtröder, Dyrskjøt.
Critical revision of the manuscript for important intellectual content: Birkenkamp-Demtröder, Dyrskjøt, Ørntoft.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2016.01.007.

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