

# The current status and clinical value of circulating tumor cells and circulating cell-free tumor DNA in bladder cancer

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**Abstract:** Urothelial carcinoma of the bladder (UCB) is a complex disease, which is associated with highly aggressive tumor biologic behavior, especially in patients with muscle-invasive and advanced tumors. Despite multimodal therapy options including surgery, radiotherapy and chemotherapy, UCB patients frequently suffer from poor clinical outcome. Indeed, the potential of diverse opportunities for modern targeted therapies is not sufficiently elucidated in UCB yet. To improve the suboptimal treatment situation in UCB, biomarkers are urgently needed that help detecting minimal residual disease (MRD), predicting therapy response and subsequently prognosis as well as enabling patient stratification for further therapies and therapy monitoring, respectively. To date, decision making regarding treatment planning is mainly based on histopathologic evaluation of biopsies predominantly derived from the primary tumors and on clinical staging. However, both methods are imperfect for sufficient outcome prediction. During disease progression, individual disseminated tumor cells and consecutively metastases can acquire characteristics that do not match those of the corresponding primary tumors, and often are only hardly assessable for further evaluation. Therefore, during recent years, strong efforts were directed to establish non-invasive biomarkers from liquid biopsies. Urine cytology and serum tumor markers have been established for diagnostic purposes, but are still insufficient as universal biomarkers for decision-making and treatment of UCB patients. To date, the clinical relevance of various newly established blood-based biomarkers comprising circulating tumor cells (CTCs), circulating cell-free nucleic acids or tumor-educated platelets is being tested in cancer patients. In this review we summarize the current state and clinical application of CTCs and circulating cell-free tumor DNA originating from blood as biomarkers in patients with different UCB stages.

**Keywords:** Urothelial carcinoma of the bladder (UCB); biomarker; liquid biopsy; circulating tumor cells (CTCs); circulating tumor DNA (ctDNA)

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## Introduction

With an estimated 430,000 newly diagnosed cases and over 165,000 deaths worldwide among both genders, urothelial carcinoma of the bladder (UCB) represents the 7th most common cancer worldwide and a significant burden of morbidity and mortality (1). The majority of patients present with non-muscle invasive UCB (NMIBC,

non-muscle invasive bladder cancer) at initial diagnosis. Although recurrence rates in NMIBC are high with 50–70% and progression to muscle-invasive UCB (MIBC, muscle invasive bladder cancer) within 5 years is observed in about 15% of patients, most patients usually are eligible for bladder preserving treatment including transurethral resection and intravesical instillation therapies (2).

However, up to a third of patients have muscle-invasive disease at primary diagnosis and up to 50% have recurrent high-grade non-muscle invasive disease or progression to muscle-invasive disease during their lifetime (3,4). Radical cystectomy (RC) with bilateral pelvic lymphadenectomy with or without perioperative chemotherapy is the standard of care in these patients (3).

Despite advances in the surgical and perioperative management, improvement of imaging techniques and progress in systemic therapies, outcomes virtually remain unchanged since decades and almost 50% of patients develop metastases and die from their disease within 5 years after surgery (5). Of importance a substantial number of patients demonstrate early progressive disease and develop metastases within the first 2 years after treatment with curative intent (6). This might be due to occult metastases growing from minimal residual disease (MRD), which remained undetectable even by modern, high resolution imaging procedures. Thus, biomarkers are urgently needed (I) to improve clinical staging as prerequisite for patient counseling and therapy guidance in UCB; (II) to detect MRD; (III) for predicting therapy response and subsequently prognosis and (IV) to enable patient stratification for multimodal treatment and therapy monitoring, respectively. Phenotypically similar tumors may harbor completely different molecular genotypes representing the individuality of each tumor and its host (7). Besides common histopathological parameters such as tumor stage and grade, genetic characterization and classification of UCB in different molecular subtypes has gained clinical interest (8). However, because of the strong heterogeneity of UCB and conflicting results of already applied targeted therapies which were based on genetic or gene expression profiling of tumor tissue, implications for targeted treatment still have to be optimized and validated in clinical trials (9). Several tissue and blood-based biomarkers have been investigated and hold the potential to unmask individual genomic, epigenetic, transcriptomic, and proteomic alterations that may explain the variable clinical course of disease (10). Biomarkers that may detect clinically relevant occult metastatic disease or MRD may help selecting patients best suited for multimodal therapy and sparing those patients, who are likely to be cured with local therapy alone, from the toxicity associated with unnecessary systemic therapy (11).

Likewise, in patients with metastatic or unresectable disease, therapeutic decisions ideally should be based on predictive biomarkers. Although standard platinum-

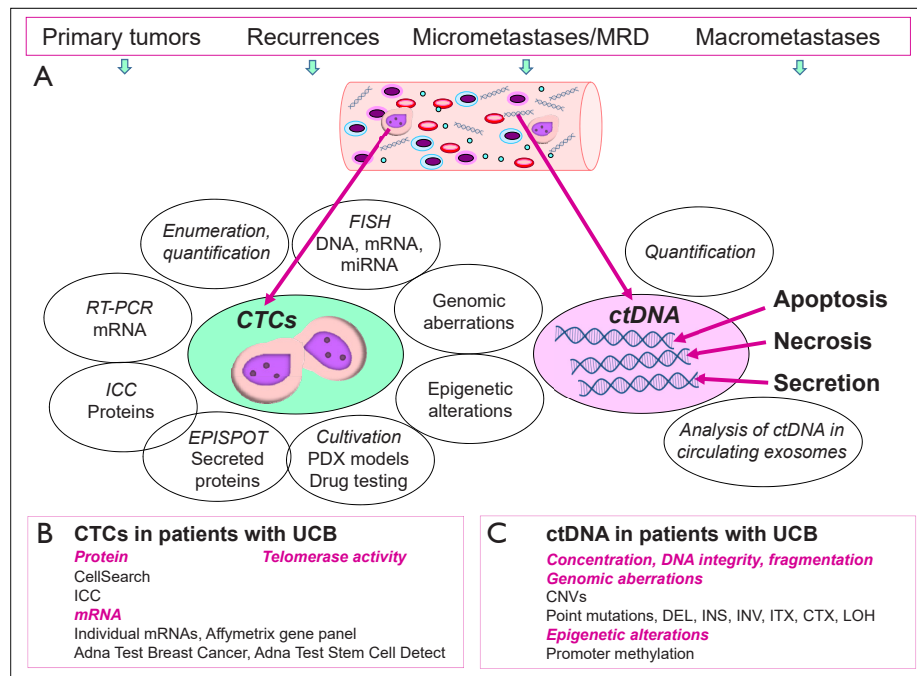
based systemic chemotherapies yield initially in reasonable response rates, a significant number of patients progresses after approximately 1 year and the optimal subsequent systemic treatment remains unsettled since second-line agents demonstrated only limited activity (12). Administration of biologically targeted agents combined with systemic chemotherapy may represent an option for advanced, metastatic UCB patients with improved response rates. Nevertheless, systemic UCB chemotherapies often cause hematologic and non-hematologic side effects with serious grade 3/4 toxicities in over 50% of patients (13). Also of importance, new immunomodulatory therapies, which demonstrated reasonable response rates in several patients, still fail in a substantial percentage of patients and are causing enormous costs (14-16).

In this review article we summarize the current state, clinical application, potential and limitations of circulating tumor cells (CTCs) and circulating cell-free tumor DNA originating from blood as biomarkers in patients with different UCB stages.

### Potential of liquid biopsy as biomarker

To date, decisions to treat cancer patients are mainly driven by characteristic features of the primary tumor and potentially its metastasis. For this reason, tumor tissue is evaluated by immunohistochemistry, fluorescence *in situ* hybridization (FISH), gene expression profiling or genomic analyses. In most cases, however, only an incomplete copy of the multitude of different facets of tumor cells can be reflected in daily routine. The situation is even more complicated when tumor disease progresses, individual tumor cell clones propagate and tumor cells acquire additional molecular aberrations over time and under treatment conditions. Identification of underlying driving forces and their elimination would require comprehensive evaluation of primary tumors, recurrences and metastases. However, restaging by evaluation of biopsies from metastases is restricted to selected easily accessible organs such as liver or skin and mostly impossible for other target organs. Thus, essential information for elimination of tumor cells in advanced stage patients is missing (17-20).

Unfortunately, conventional serum tumor markers are inaccurate and frequently fail because of low sensitivity and specificity. Accordingly, new biomarkers capable to real time record and monitor the actual disease state as well as to predict prognosis, therapy response and resistance detectable without invasive intervention are urgently



**Figure 1** Detection and characterization of circulating tumor cells and circulating tumor DNA, released from primary tumors, recurrences, minimal residual disease and metastases. (A) Several methods to detect and characterize CTCs on protein, mRNA and genomic level have been established and already applied for different tumor entities. Moreover, isolation of viable CTCs is possible and enables cultivation and patient derived xenograft models (PDX). Secretion of proteins by CTCs can be determined by the EPISPOT (EPithelial ImmunoSPOT) assay. Epigenetic alterations can be detected both on CTCs and cell-free ctDNA; (B) approaches and markers for CTC detection already applied for patients with UCB; (C) approaches for ctDNA detection and markers already established for patients with UCB. FISH, fluorescence in situ hybridization; RT-PCR, reverse transcriptase PCR; ICC, immunocytochemistry; CNV, copy number variation; DEL, deletion; INS, insertion; INV, inversion; ITX, intra-chromosomal translocations; CTX, inter-chromosomal translocations.

needed in “precision medicine”. There is growing evidence that liquid biopsies have the potential to fill this gap (17-20).

For UCB urine intuitively seems to be the optimal resource, because of the direct contact to tumor tissue and tumor cell release (21). For that reason analyses of urine samples (i.e., urine cytology) are routinely performed in daily clinical practice. Urine cytology combined with FISH as well as determination of tumor-related antigens (bladder tumor antigens, NMP22) and of genomic aberrations in DNA isolated from urine has recently been improved mainly for diagnostic implications, but still lacks sufficient clinical validation (22,23). Suggested from a very recent study by Patel *et al.*, mutant DNA detected in urine also has the potential to serve as biomarker for monitoring of neoadjuvant therapy (NT) (21). However, analyses of urinary samples will not be in the scope of our current review.

In this article, we focus on blood as liquid biopsy

with special emphasis on CTCs and cell-free circulating tumor DNA (ctDNA) (Figure 1). Blood samples are easily accessible during the course of the disease and serial collection allows for long term observation of cancer patients. While CTCs have to be enriched from whole blood, buffy coats or enriched peripheral blood mononuclear cells immediately after blood drawing, ctDNA is isolated from serum or plasma samples which can be stored after collection until processing. Thus, both potential biomarkers have advantages and disadvantages and the most suitable one has to be chosen according to the question to be addressed and the particular study design (24,25).

### CTCs

CTCs are rare cells and especially in early tumor stages their concentration is very low thus requiring pre-enrichment to eliminate erythrocytes and the majority of

leukocytes (26). Several enrichment methods relying either on physical properties such as cell size, plasticity, density or dielectrophoretic mobility or on the expression of antigens captured by high affinity magnetic particle-coated antibodies have been established yet. However, selecting the most effective one depending on the particular study design and planned down-stream analyses is challenging (26). Also for the detection of CTCs within the enriched cells manifold approaches are available and identification of tumor-cell specific transcripts by reverse transcriptase PCR (RT-PCR) or antigens by immunocytochemistry enable distinguishing CTCs from still contaminating blood cells (26,27). Because of the lack of tumor cell-specific markers, still epithelial cell properties represent the gold standard as surrogate markers to trace CTCs. Advantages and disadvantages of currently available detection platforms are discussed in several recent review articles (26-29).

Moreover, numerous accompanying approaches have been established for phenotypical and molecular characterization of CTCs to complement enumeration and quantification (*Figure 1*). Expression of therapeutically relevant proteins such as HER2 (human epidermal growth factor receptor), EGFR (epidermal growth factor receptor), ER (estrogen receptor), AR (androgen receptor) or PSMA (Prostate Specific Membrane Antigen) investigated by immunocytochemistry has been reported for CTCs derived from breast, colorectal and prostate cancer (30-35). Also, transcriptomic profiles were commonly described for enriched CTC populations (36,37), and even at single CTC level (38). Moreover, different approaches to explore genomic aberrations of CTCs have been established including FISH and PCR-based techniques. For single CTC analyses, propagation of DNA by WGA (whole genome amplification) is required, before mutational and CNV (copy number variations) analyses by Sanger sequencing, CGH (comparative genomic hybridization) and NGS (next generation sequencing) can be applied (35,39-41). Now clinical studies have to verify whether genomic aberrations of CTCs reflect the actual genomic landscape of the disease and therapy response or resistance, respectively, more accurately than genomic profiles of the corresponding primary tumors.

The successful isolation of viable CTCs has paved the way for a variety of down-stream investigations such as detection of secreted proteins, cultivation, xenograft and PDX (patient-derived xenografts) models which enable drug testing and further experiments to identify new therapeutic targets (*Figure 1*) (42-47).

## CTCs in patients with UCB

### Detection of CTCs by immunological methods

Meanwhile, several studies investigated the clinical relevance of CTC in UCB, however, the comparison of results from different groups is only feasible if similar detection techniques were applied (*Table 1*). The only standardized method available thus far is the CellSearch system, which has been cleared by the FDA (Food and Drug Administration) for the analysis of blood samples from patients with metastatic breast, prostate and colorectal cancer (88-90). In brief, in the first step CTCs are enriched from whole blood (either peripheral or from tumor-draining veins) by anti-EpCAM (epithelial cell adhesion molecule) antibodies coated to ferrofluid. In a second more specific step, identification of CTCs is performed by immunofluorescence with anti-keratin (KRT) antibodies and negativity for the common leukocyte antigen CD45. Subsequently, scanning of cells is carried out by an automated fluorescence microscope and suspicious images are presented in an image gallery for visual evaluation. High specificity and reproducibility of this system has been demonstrated in several multicenter studies (88,91,92). Although not yet included in the above-mentioned FDA approval, CTCs derived from patients with UCB can also be detected with the CellSearch system (*Table 1*). However, the numbers of enrolled patients are mostly low and no large multicenter clinical trials incorporating CTC analyses have been initiated, yet.

Using the Cell Search system, in UCB patients with non-metastatic disease CTCs were detected in about 20-30% of patients before surgical intervention or chemotherapy (*Table 1*) (48-53,55,57,59,61). The presence of CTCs correlated to FDG-PET-CT (2-deoxy-2-<sup>18</sup>F-fluoro-d-glucose positron emission tomography/computer tomography) imaging (48), but was not predictive for extra-vesical or node-positive disease (61). Interestingly, in most studies CTC detection was not associated with clinicopathological parameters.

In cohorts of patients with NMIBC including exclusively patients with high grade (G3) pT1 tumors, about 20% of patients harboured CTCs. In these studies, CTC detection was significantly associated with a reduced time to first recurrence and time to disease progression, defined as upstaging of the disease or appearance of distant metastases (49,55).

For patients with MIBC or recurrent high risk NMIBC treated with RC, the proof of CTCs had prognostic relevance regarding inferior recurrence-free, cancer-specific and overall survival (RFS, CSS, OS). The presence of

**Table 1** Clinical relevance of circulating tumor cell detection in patients with urinary carcinoma of the bladder

Patients, time point of blood collection	No. of pts	Method	CTC positivity (range*)	Association of CTCs with tumor/patient characteristics and clinical outcome	Ref.
CellSearch and immunocytochemistry					
UCB (nm, m), before surgery; if NT was applied: before NT and before RC (if CTC+) Palliative: before treatment, after 3–4 cycles (if CTC+)	78 (RC); 10 (palliative)	CellSearch	17/88 (19.0%) (1–105, median: 3); 14/78 RC (18%); 3/10 palliative (30.0%)	FDG-PET CT, but not PET-CT, PFS in pts. treated with RC, multivariate	(48)
High risk NMIBC, pT1G3 (TURBT), before TURBT	101 (CellSearch); 54 (CELLlection)	CellSearch; CELLlection Dynabeads	20/101 (19.8%); 24/54 (44.4%)	Follow up: 28 months, TFR, TTP	(49)
MIBC, before RC	135 without AC, 50 with AC	CellSearch	41/185 (22.0%) (1–163, median: 1)	Median follow up: 31 months, RFS, CSS, OS in patients without AC. No association in patients with AC	(50)
MIBC, pure and variant UCB histology, before RC	Variant histology (n=47) most frequently of SCC; pure UCB (n=141)	CellSearch	30/141 (21.3%) pure UCB; 12/47 (25.5%) UCB + variant histology	Median follow up: 25 months, presence of CTCs and non-squamous differentiation: RFS and CSS, multivariate: presence of CTCs, but not variant histology: RFS and CSS	(51)
pT <sub>a</sub> low and high grade, pT <sub>1</sub> low and high grade, and pT <sub>2</sub> high grade, before and after TURBT	8	CellSearch	2/8 (25.0%) [1–4] before TURBT, 3/8 (37.5%) [1–12] after TURBT	Increase of CTC counts postoperatively in 3 pts. (0–1; 4–5; 0–12)	(52)
cT <sub>2</sub> -T <sub>4</sub> , N <sub>+</sub> /–, M <sub>+</sub> /–, before Chemo, after Chemo (if CTC+)	26	CellSearch	N0-2 M0: 3/16 (19.0%) (1–1); N3 or M1: 5/10 (50.0%), 4/5 with >5 (range 7–70)	Decline of CTC counts during Cisplatin-based therapy	(53)
Peripheral vein and inferior vena cava, before and after TURBT	NMIBC (n=6); MIBC (n=10)	CellSearch	NMIBC: 1/6 (16.7%) increase of CTC counts in vena cava blood and PB; MIBC: 6/12 (50.0%), increase of CTC counts in 5/6 (vena cava) and 4/6 (PB)	Tumor cell release during TURBT	(54)
High risk NMIBC, pT <sub>1</sub> G <sub>3</sub> , TURBT candidates, followed by BCG therapy, before TURBT	102	CellSearch	20/102 (20%) (1–50, median: 1)	Median follow up: 24.3 months, female gender, tumor size, CIS, multi-focality, lymph vascular invasion and appearance of distant metastases, PFS, Bivariate: TFR, TTP and MFI; Multivariate: TFR, TTP	(55)
NMIBC, before TURBT, pT <sub>a</sub> and pT <sub>1</sub> , G <sub>1</sub> -G <sub>3</sub>	44	CellSearch	8/44 (18%), (1–3, mean: 1.5)	Follow up: 24 months, TFR, T stage and concomitant CIS	(56)

**Table 1** (continued)



Table 1 (continued)

Patients, time point of blood collection	No. of pts	Method	CTC positivity (range)	Association of CTCs with tumor/patient characteristics and clinical outcome	Ref.
UCB (nm), before RC	100	CellSearch	Preoperatively: 23/100 (23.0%) (1–100, median: 1); strong HER2 expression in CTCs in 3/22 patients	DFS, CSS, OS, also multivariate. No association with clinic-pathological features	(57)
Local and m UCB, pre- and postoperatively	44	CellSearch	Preoperatively: 5/28 (18.0%) [1–6], postoperatively: 0/2, metastatic: 7/14 (50.0%) [1–177]; Confirmation of malignant origin by FISH with Urovision probes	Follow up: 337 days, all metastatic CTC-positive patients and 3/7 (43%) metastatic CTC-negative patients died	(58)
UCB (nm, m), before RC	50; 5	CellSearch	15/50 (30.0%); 5/5 (100.0%)	Median follow up: 1 year for 53 pts., CSS, PFS, OS	(59)
UCB (nm, m), before Chemo	16; 20	CellSearch	0/16; 11/20 (55.0%)	Higher CTC counts in pts. with multiple metastases compared to single metastasis	(60)
pT0–pT4, before RC	43	CellSearch	9/43 (21.0%) (1–9, median: 1)	No association with pT and not a robust predictor of extra-vesical or node-positive disease	(61)
pT1–pT3b, before and during RC	5	CellSearch	1/5 (pT3 G3–4) before and during RC; 2 CTCs/25 mL before; 1 CTC/25 mL during RC	No increase of CTC counts by RC	(62)
UCB (m), before Chemo	33	CellSearch	14/33 (42.0%) (0–87)	Higher CTC counts in pts. with multiple metastases compared to single metastasis	(63)
UCB and cancer of urethra, before surgery	12 non-metastatic; 14 metastatic	CellSearch	0/12; 8/14 (57.1%, mean: 9.21)	CTC detection significantly more frequent in M1 versus M0 patients	(64)
MIBC (nm, m), at diagnosis or after Chemo	4 MIBC, 15 metastatic	EpiScience; ICC	20/25 (80.0%) inclusive of KRT <sup>+</sup> CTCs (13/25, 52.0%), KRT CTCs (14/25, 56.0%), KRT <sup>+</sup> CTC Clusters (6/25, 24.0%), and apoptotic CTCs (13/25, 52.0%). 7/25 (28.0%) PD-L1 <sup>+</sup> CTCs; 4 with exclusively KRT <sup>+</sup> /CD45 <sup>+</sup> /PD-L1 <sup>+</sup> CTCs	FISH for confirmation of malignant origin, pts. with high PD-L1 <sup>+</sup> /CD45 <sup>+</sup> CTC burden and low burden of apoptotic CTCs had worse OS	(65)
128 carcinoma patients (prostate, bladder, renal), before, during and after surgery	34 UCB	Ficoll, CD45-based leukocyte depletion by autoMACS; KRT, ICC	52/128 (40.6%)	CTC detection rates increased in the order prostate carcinoma < renal cell carcinoma < bladder cancer	(66)
pTa–pT4; nm UCB, before and after surgery, prior to Chemo	pTa–pT1: 20; pT2–pT4: 11	Ficoll, ICC; KRT	1/31 (3.2%)	Only one patient with metastatic disease	(67)

Table 1 (continued)

Table 1 (continued)

Patients, time point of blood collection	No. of pts	Method	CTC positivity (range)	Association of CTCs with tumor/patient characteristics and clinical outcome	Ref.
<b>Telomerase activity-based assays</b>					
MIBC, before and after therapy	2	Telomerase-based CTC assay	#1: 0 before and 202 CTCs/mL after radiotherapy; #2: 631 CTCs/mL before and decrease to 194 CTCs/mL after radiotherapy	CTCs might support tracking of the disease	(68)
High grade, muscle invasive or m UCB	30 UCB; 17 healthy controls	TRAP-Assay	27/30 pts. (90.0%) with high grade, MIBC or m UCB, but in none of 17 healthy controls	Not analyzed	(69)
<b>Reverse transcriptase polymerase chain reaction (RT-PCR)</b>					
T1G3 NMIBC, before TURBT	54	CELLlection Dynabeads survivin ( <i>BRC5</i> ), <i>KRT 8</i> , <i>CD45</i>	CTCs: 24/54 (44.0%), 22/24 (92.0%) expressed <i>BRC5</i> mRNA	Median follow up: 9 years, presence of CTCs and <i>BRC5</i> mRNA in CTCs associated with DFS and CSS	(70)
UCB (m), treatment with MVAC	31	Adna Test Prostate Cancer Select Kit	At baseline: 17/31 (54.8%); After 2 cycles chemo: 17/26 (65.4%)	No association of CTCs with objective response to MVAC, CTC dynamic changes better predictive for 3 year PFS and OS than single point CTC measurements	(71)
UCB, before RC	59	Ficoli; <i>KRT 7</i>	23/59 (38.9%)	Median follow up: 42 months, T and N, increased risk for recurrence, decreased CSS, OS, uni- and multivariable	(72)
NMIBC and MIBC (nm): before surgery m: before or during Chemo	83 pts., 29 controls	Adna Test Breast Cancer Detect; Adna Test Stem Cell Detect	Epithelial transcripts: controls (A), 2/29 (6.9%); NMIBC (B), (6.7%); cM0 MIBC (C) (15%); cM1 MIBC (D) (18.7%). Stem cell-specific transcripts: A, 10.3%; B, 10.0%; C, 22.5%; D, 31.3%. EMT-related transcripts: A, 3.5%; B, 3.3%; C, 15.0%; D, 18.7%	Stem cell and EMT-specific transcripts correlate with clinical stage of disease	(73)
History of UCB newly diagnosed UCB, before surgery or any other intervention	169 pts., 39 controls	Lysis of erythrocytes; <i>KRT 19</i> , <i>KRT 20</i> , <i>EGFR</i>	More CTCs in pts. with newly diagnosed UCB than in those with history of UCB and normal controls	<i>EGFR</i> and <i>EGFR</i> plus <i>KRT 19</i> and <i>KRT 20</i> mRNAs correlated to histological grade and CIS	(74)
≥ cT1c UCB, before and after TURBT	51	PAXgene Blood RNA Kit, 14 transcripts	<i>EGFR</i> , Collagen alpha-1(I) chain, Mast/stem cell growth factor receptor ( <i>KIT</i> ) and <i>CD47</i> mRNAs, significant differences in gene expression between controls and cancer patients	No evidence for increased tumor cell release by TURBT	(75)

Table 1 (continued)

Table 1 (continued)

Patients, time point of blood collection	No. of pts	Method	CTC positivity (range)	Association of CTCs with tumor/patient characteristics and clinical outcome	Ref.
NMIBC (T1G3), before TURBT	54	CELLlection; CD45, KRT 8, BRC5	24/54 patients (44.0%); 92.0% of CTCs BRC5-positive	Independent for DFS	(76)
NMITCC, MITCC, before surgery	59 NMITCC; 9 MITCC; 22 healthy controls	Ficoll; Nested RT-PCR KRT 20	pT1a-T3: 19/57 (33.3%); superficial TCC: 1/9 (11.0%)	No correlation to pathological state	(77)
UCB	19	TNC and EGFR	11/19 (58.0%) TNC mRNA; worse prognosis in pts. with low-stage disease, 11/19 (58.0%)	EGFR and TNC mRNAs: DFS and RFS	(78)
UCB	40 advanced stage UCB; 22 without evidence of disease at time of assay	Lysis of erythrocyte, Nested RT-PCR, UPK Ia, Ib, II and EGFR	8 pts. with recurrent disease positive for UPK Ia/UPK II mRNAs, EGFR mRNA alone and in combination with UPKs inferior to UPK Ia/UPK II mRNAs	Median follow up: 15 months, combinations of urothelial markers superior to single urothelial or epithelial markers in detecting CTCs in UCB pts	(79)
UCB and healthy controls	16 UCB; 40 UCB (validation cohort); 27 healthy controls	Affymetrix U133 Plus 2.0 GeneChip	Differential expression of: IGFBP7, SNX16, CSPG6, CTSD, CHD2, NELL2, TNFRSF7 mRNAs	Gene expression profile of CTCs: distinguishing of bladder cancer from other types of genitourinary cancer and healthy controls	(80)
UCB, before surgery	38; 18 non-cancer pts.	Buffy coat, nested RT-PCR for MUC7	No MUC7 mRNA in control group, 18 of 38 (47.4%) UCB	Higher grade of differentiation	(81)
UCB, before RC or TURBT	11 before RC; 9 before TURBT; 25 healthy controls	KRT 20	Preoperatively: 2/20 (10.0%); post-operatively: 4/20 (20.0%)	Preoperatively: advanced stage, postoperatively: 3 pTaNXM0 and pT1NXM0, 1pT3aN2M0	(82)
TCC, before TURBT or RC	27 TCC: 6 superficial (T1s-T1-N0M0), 10 MITCC (T2-4 N0M0), 3 pN1-2 M0, 8 metastatic (M1), 30 healthy controls	Succinyl-linked gelatin-based separation of erythrocytes, ACTB, EGFR, KRT 19, 20, UPK-II	EGFR mRNA: 20/27 (74.0%), KRT 19 mRNA: 11/27 (41.0%), KRT 20 mRNA: 4/27 (15.0%), UPK II mRNA: 10/27 (37.0%)	Follow up: 20 months, EGFR mRNA in the majority of metastatic patients and non-metastatic patients: emergency of relapse, independent of clinical stage of tumors at surgery	(83)

Table 1 (continued)



Table 1 (continued)

Patients, time point of blood collection	No. of pts	Method	CTC positivity (range*)	Association of CTCs with tumor/patient characteristics and clinical outcome	Ref.
NMIBC and MIBC	40	Enrichment of PBMCs, Nested RT-PCR, <i>KRT 20</i>	<i>KRT 20</i> mRNA: 9/40 (22.5%); 0/13 superficial, 4/21 (19.0%) regionally invasive and 5/6 (83.0%) metastatic (P=0.0002 in chi 2 test)	Haematogenic dissemination in invasive UCB, but rarely in superficial UCB	(84)
UCB	50 non-metastatic and 10 metastatic, 10 healthy controls	Nested RT-PCR, <i>UPK II</i>	<i>UPK II</i> mRNA: 2 pts with metastatic TCC without chemotherapy and 1/8 with chemotherapy, no <i>UPK II</i> in non-metastatic pts. and controls	<i>UPK II</i> mRNA associated with metastatic spread	(85)
UCB	9 non-metastatic and 3 metastatic	Nested RT-PCR: <i>UPK II</i>	<i>UPK II</i> mRNA in all 3 metastatic TCC, but not in 9 TCC with non-metastatic TCC nor in 3 healthy volunteers	<i>UPK II</i> mRNA may provide helpful information in the diagnosis and management of TCC	(86)
UCB	39 pts. and 9 controls	Enrichment of PBMCs, <i>KRT 19</i> by nested RT-PCR, confirmation by Southern Blotting	No <i>KRT 19</i> mRNA in controls, <i>KRT 19</i> mRNA in 3/10 UCB (2 metastatic, 1 MIBC M0)	<i>KRT 19</i> mRNA by nested RT-PCR: detection of micrometastasis, evaluation of therapeutic effects and prediction of prognosis	(87)

\*; number of CTCs/7.5 mL; #, patient-ID. UCB, urothelial carcinoma of the bladder; Pts, patients; PB, peripheral blood; PBMN, peripheral blood mononuclear cells; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; nm, non-metastatic; m, metastatic; SCC, squamous cell carcinoma; CIS, carcinoma in situ; TNM, tumor stage, nodal state, distant metastasis; p, pathological; c, clinical; G, grading; X, not known; CTC+, positive for circulating tumor cells; PFS, progression free survival; DFS, disease-free survival; OS, overall survival; RFS, recurrence-free survival; CSS, cancer specific survival; TFR, time to first recurrence; TTP, time to progression; MFI, metastasis-free interval; RC, radical cystectomy; AC, adjuvant chemotherapy; Chemo, chemotherapy; MVAC, methotrexate/vinblastine/doxorubicin/cisplatin; NT, neoadjuvant therapy; TURBT, transurethral resection of bladder tumors; BCG, bacillus calmette guerin; FDG-PET/CT, fluorodeoxyglucose-positron emission tomography/computed tomography; TRAP, telomerase repeat amplification protocol; EMT, epithelial-mesenchymal transition; MACS, magnetic cell isolation and cell separation; ICC, immunocytochemistry; KRT, keratin; HER2, human epidermal growth factor receptor; EGFR, epidermal growth factor receptor; PD-L1, programmed death-ligand-1; BRC, survivin; MUC, mucin; KIT, tyrosine kinase 117; TNC, tenascin C; IGFBP7, insulin-like growth factor-binding protein 7; SNX16, sorting nexin 16; CSPG6, chondroitin sulfate proteoglycan 6; CTSD, cathepsin D; CHD2, chromodomain helicase DNA-binding protein 2; NELL2, Nell-like 2; TNFRSF7, tumor necrosis factor receptor superfamily member 7; CD, cluster of differentiation.

CTCs was confirmed as independent predictive factor for early systemic disease and OS by multivariable analysis that adjusted for standard clinicopathological parameters (57,59). Of note, another study demonstrated that considering only patients who were spared from adjuvant chemotherapy after RC (*Table 1*), presence of CTCs prior to RC had an independent prognostic value predicting RFS, CSS and OS (50). The authors concluded that proof of CTCs potentially may identify patients in need of adjuvant chemotherapy and therefore may be a useful marker for patient counseling and decision-making. In contrast, in the same study, the presence of CTCs did not have prognostic relevance for RFS, CSS and OS, respectively, in patients who did receive adjuvant chemotherapy (50). This effect may either be a result of the potential of adjuvant chemotherapy on CTCs or due to the unfavorable general prognosis in UCB patients scheduled for adjuvant chemotherapy. In a further study the same group of authors found that CTCs are detectable in pure UCB and in UCB with variant histologies. CTCs still represented an independent prognostic factor for clinical outcome when results were adjusted for pure UCB or those with variant histology among other clinicopathological parameters (51).

A significantly higher number of CTCs was detected in patients with metastatic UCB ranging from 30% to 100% in different studies (*Table 1*) (48,53,58-60,63,64,93).

The CellSearch system only ensures detection of CTCs that are positive for EpCAM and KRT. Thus, tumor cells that have lost their epithelial features, e.g., in the course of epithelial-to mesenchymal transition (EMT) cannot be detected by this device. However, as evidenced recently by using the Epic Sciences platform, also KRT-negative CTCs are present in the circulation from UCB patients. Here, the malignant character of KRT-negative CTCs was confirmed by chromosomal aberrations detected by FISH and CNVs (65). Another study also reported usage of EpCAM-independent enrichment of CTCs by depletion of CD45-positive cells using magnetic cell separation and subsequent immunocytochemistry with anti-KRT antibodies. The CTC detection rate in this study was higher than those described in studies using the CellSearch system (*Table 1*) (66). Lower detection rates were observed when applying anti-KRT antibodies in ICC on PBMCs enriched by density gradient centrifugation (67). Moreover, application of assays based on the activity of the enzyme telomerase that is thought to be expressed only in tumor and not in normal cells might

help to identify CTCs that have down-regulated epithelial cell properties (*Table 1*) (68,69).

Currently efforts are spent for establishing phenotypical markers valuable to stratify UCB patients for individualized targeted therapies. In this context the detection of HER2 expression of CTCs has to be mentioned. Discordances detected between the HER2 status of CTCs and that of the corresponding primary tumors suggest that a subgroup of patients with HER2-positive CTCs likely profits from a HER2 targeting therapy (57). Although still not conclusively shown in large studies treatment of HER2-positive MIBC, who were not candidates for RC, by daily radiation combined with paclitaxel and trastuzumab revealed to be an effective strategy with high completion rate and moderate toxicity (94). On the other site, the presence of HER2-negative CTCs in patients with HER2-positive corresponding primary tumors might be responsible for the failure of HER2-targeting therapies in a considerable number of patients (57). In patients with metastatic UCB, a high concordance of the HER2 status between CTCs and primary tumors could be observed (93).

Moreover, there is an urgent necessity to identify biomarkers predictive of response to immune checkpoint inhibitors which have already been FDA-approved for the treatment of patients with metastatic UCB (95). Anantharaman *et al.* were the first to display programmed cell death-ligand 1 (PD-L1) expression on metastatic UCB-derived CTCs (65), however clinical value of PD-L1 analysis on CTCs remains to be conclusively shown in large clinical immune checkpoint inhibitor studies. Interestingly, PD-L1 expression of KRT-positive and KRT-negative CTCs has been demonstrated in this study.

#### **Detection of CTCs by RT-PCR**

Besides methods allowing also assessment of morphological characteristics and gene expression levels of individual CTCs, assays to detect CTCs by tumor cell specific transcripts from enriched CTCs have been established. Also here, initial enrichment of CTCs is required and commonly achieved by anti-EpCAM antibodies coupled to magnetic beads (70,71,73) or by marker-independent approaches such as density gradient centrifugation (77). Moreover, RNA was isolated from whole blood with preceding red blood lysis in some other studies (*Table 1*) (74,79). However, standardized assays and clinically validated cut-off values are still missing.

Once enriched, evidence of CTCs will be provided by highly sensitive RT-PCR approaches and quantification of tumor-cell-specific transcripts occurs by quantitative RT-PCR. The extraordinarily high sensitivity to trace even low

abundant tumor cell transcripts might be accompanied by less specificity due to very low intensity or illegitimate expression of the gene of interest in contaminating leukocytes (96,97). Nevertheless, there are a remarkable number of studies demonstrating clinical value of CTCs detected by RT-PCR, e.g., in breast, prostate and lung cancer. In these studies either individual tumor cell transcripts or gene expression profiles were detected (17,98-102).

*Table 1* summarizes results for CTC detection by RT-PCR obtained for UCB patients. Early studies for mainly small numbers of patients aimed to detect transcripts specific for *KRT19* and *UPK II* (uroplakin II) demonstrated that CTCs originating from UCB can support detection of metastasis including micrometastases, evaluation of therapeutic effects and prediction of prognosis (85-87). *KRT20*-specific transcripts expressed by luminal subtypes of UCB revealed to be indicative of hematogenous tumor cell dissemination in MIBC, but not in superficial tumors (84) or prior to transurethral resection of the bladder tumor (TURBT) for advanced tumor stage (82). Studies published by Leotsakos *et al.* and Gazzaniga *et al.* (2001 and 2005) provided evidence for prognostic relevance of *EGFR*, *KRT19*, *20*, *UPKII* and *TNC* (tenascin C) transcripts (74,78,83). Thus, detection of *EGFR* and *TNC* mRNAs was associated with reduced DFS and RFS for UCB patients. Osman *et al.* showed that the combined detection of different transcripts such as *UPKI*, *II* and *EGFR* or the examination of gene expression profiles by Affymetrix microchips was superior to single urothelial or epithelial markers in detecting CTCs (79,80). In patients with *NMIBC*, *KRT8*-, and *BRC5* (survivin)-specific transcripts were predictive of reduced DFS (76) and DFS and CSS (70), respectively.

Applying the commercially available Adna Test Breast Cancer, or stem cell Test for blood samples from *NMIBC* and non-metastatic or metastatic MIBC, Todenhöfer *et al.* assumed that epithelial cell-, but also stem cell- and EMT-specific transcripts are indicative for the clinical stage of the disease (73). Moreover, the prevalence of *KRT7* mRNA detected before RC was associated with tumor and lymph node state, with increased risk of recurrence and independently with reduced CSS and OS of CSS of UCB patients (72). However, although no association of CTCs detected with the Adna Test Prostate Cancer—applied for UCB patients—with objective response to treatment of metastatic UCB (methotrexate, vinblastine, doxorubicin, cisplatin) could be observed, dynamic changes of CTCs were more predictive for progression-free survival (PFS) and OS than single point measurements (71).

In conclusion, prognostic relevance of CTC detection by RT-PCR could be demonstrated in several studies on still small patient cohorts. Application of those tests in large multi-centric studies is hindered by the low stability of RNA complicating shipment and storage of samples. However, similar to CellSave tubes for CellSearch strong efforts are made to develop blood-drawing tubes ensuring shipment and storage of blood samples for RT-PCR analyses without losing significant information. Moreover, from the variety of assay formats and UCB-associated transcripts the best suited have to be chosen to establish a standardized approach using a pre-defined UCB-specific panel of transcripts. Then multi-center clinical trials for monitoring systemic therapies can be initiated with serial blood investigations during follow up observations of the patients.

#### **Release of tumor cells by surgical interventions**

There is growing interest in addressing the question whether surgical or other manipulations on tumor tissue are accompanied by tumor cell release which gives rise to later development of recurrences or metastasis.

For patients with *NMIBC*, conflicting results from only three studies enrolling small numbers of patients are available. While tumor cell release was observed by Blaschke *et al.* and Engilbertsson *et al.* applying the CellSearch system (*Table 1*) (52,54), an RT-PCR-based study did not provide evidence for tumor cell release by TURBT (75). Conclusively, only serial CTC measurements at defined time points within prospective long term-follow up studies on large patient cohorts can uncover whether increased CTC counts by surgical intervention or other tumor manipulations are associated with an increased risk of disease recurrence and metastases. Additionally, strong efforts have to be done by molecular approaches and functional studies to identify metastasis-initiating cells among these tumor cells as well as to discover potential metastatic seeding of released tumor cells. Importantly, future studies will investigate if improvements in operation techniques might have the ability to avoid tumor cell spreading.

#### **Detection of circulating cell-free tumor DNA (ctDNA) in patients with UCB**

During recent years, circulating cell-free tumor DNA (ctDNA) has gained attraction as candidate biomarker with potential to improve diagnostics, detection of MRD and therapeutic monitoring of tumor disease. Typical size of ctDNA fragments is 160 to 180 base pairs similar to the length of nucleosome-protected DNA in apoptotic

cells (103). Although DNA can be released from all kinds of cells by apoptosis, necrosis or even by active secretion and therefore is detectable in different body fluids such as blood, urine, sputum, or cerebrospinal fluid, results from different studies indicate that the concentration of cell-free DNA is higher in tumor patients compared to healthy individuals (103). However, increase of concentration can also be due to inflammatory processes, exercising or non-tumor diseases, thus requiring additional markers to discriminate between normal and tumor-derived DNA (103). As genomic or epigenetic aberrations are key features of tumor cells, covering somatic alterations in cell-free DNA (cfDNA) is indicative of the presence of cell-free tumor DNA (ctDNA) (*Figure 1*). In the pre-NGS era of circulating DNA investigations, instability in frequent microsatellite markers was used to identify loss of heterozygosity (LOH) as proof of tumor cell origin or hypermethylation of promoter regions regulating tumor suppressor or other tumor-related genes in cell-free DNA (104). Later on, technological advances in PCR and NGS approaches prepared the ground for the present capability to comprehensively analyse tumor-originating DNA accounting for less than 0.1% of total cfDNA in patients with early cancer and up to 10% in advanced stage cancer in plasma or serum (25,103,105,106).

Compared to CTCs, investigation of ctDNA has the advantage that plasma/serum samples can be long-term stored and easily be collected until final analysis, making ctDNA analyses very attractive for multicenter studies with complex and standardized assays centrally applied. The challenge of ctDNA research is to find marker alterations out of the diversity of potential genomic aberrations suitable to follow the individual tumor patient. New techniques including massive parallel NGS to explore high numbers of genes and sensitive PCR approaches to trace single mutant molecules are established, however are not amenable for daily routine applications yet (25,103).

To date, personalized ctDNA assays based on comprehensive genomic evaluation of the primary tumors are in the centre of attention and have already been approved in smaller pilot studies (107-109). Mostly performed by highly sensitive digital droplet PCR (ddPCR), personalized assays enable direct reflection of individual tumor-specific aberrations in plasma or serum samples. Moreover, circulating extracellular vesicles (exosomes, *Figure 1*) suggested to carry and transfer tumor-derived DNA (110) hold promise as informative biomarkers in cancer patients (111), but will not be discussed in this article.

### Detection of ctDNA by epigenetic alterations in patients with UCB

Tumor suppressor genes can be inactivated by hypermethylation of CpG islands in their promoter regions (104). Using methylation-sensitive/specific PCR (MSP), aberrant DNA methylation of promoter regions from a multitude of tumor-associated genes in serum-DNA could be detected in several studies (*Table 2*). The most prominent analyzed gene was *p16INK4a*, encoding a tumor-suppressing cyclin-dependent kinase inhibitor which is frequently inactivated in cancer of various organs including bladder by mutations, LOH or promoter hypermethylation (126,127). As opposed to CTCs, detection of promoter hypermethylation in circulating cfDNA was frequently associated with higher tumor stages and poor differentiation (113,117,119). However, Hauser *et al.* did not find any association of promoter methylation in cfDNA with clinicopathological parameters (115). Instead, methylation levels at each site and number of methylated genes was higher in UCB patients compared to healthy controls (115) and methylation in promoter regions from selected genes distinguished UCB patients from healthy controls (119). Moreover, proof of methylation in the promoter regions of H-Cadherin-13 (*CDH13*) (117) and Protocadherin-17 (*PCDH17*) (113) genes were of prognostic relevance for a shorter OS.

### Profiling of genomic aberrations in plasma or serum-derived circulating DNA isolated from patients with UCB

As evidenced by microsatellite-based PCR analysis, LOH was frequently detected in serum and plasma collected from patients with UCB (*Table 2*). Frequently affected microsatellites were located on chromosomes 17p and 9p obviously influencing the activity of tumor suppressor genes such as *p53* and *p16/p14* (122,123). Reduced DFS (122) and higher risk of NMIBC for progression to MIBC (123) was associated with LOH in these microsatellite markers (*Table 2*).

Technical advances mainly in NGS- and PCR-based techniques have provoked enormous progress towards clinical application of ctDNA research findings (103). Very recently, Patel *et al.* and Soave *et al.* identified CNVs and mutations in tumor-related genes by NGS and multiplex ligation-dependent probe amplification (MPLA), respectively in ctDNA from patients with non-metastatic UCB (21,112). Moreover, frequently mutated genes were *TP53* and *PIK3CA*, known to inactivate the tumor suppressor TP53 and to activate the oncoprotein PIK3CA, respectively (21).

**Table 2** Clinical relevance of circulating tumor DNA detected in blood (serum/plasma samples) from UCB patients

Patients, time point of blood collection	No. of pts.	Methods	Genomic aberrations	Association of ctDNA with tumor/patient characteristics and clinical outcome	Ref.
MIBC, NT	17 (248 samples)	SNVs, CNVs mutDNA by Tagged-Amplicon and shallow Whole Genome- Sequencing, UCB-specific sequencing panel, sampling prior to each chemotherapy session	Mutant DNA in 35.3% of pre-NT plasma	Follow up: up to 742 days after NT; mutations found in TURBT samples also found in plasma; pre-NT mutDNA: no correlation to response; less mutant DNA in plasma compared to urine; more mutDNA positive samples in pts. who recurred; most frequent mutated genes: <i>TP53</i> and <i>PIK3CA</i>	(21)
NMIBC, MIBC, before RC	72; 18 controls	MLPA, serum and plasma; 43 chromosomal regions containing 37 genes	35/72 (48.6%) CNV, median CNV count: 2, CNVs in <i>CDH1</i> , <i>ZFH3</i> , <i>RIPK2</i> , <i>PEN</i>	CNVs in <i>TSG1</i> , <i>RAD21</i> , <i>KIAA0196</i> , <i>ANXA7</i> , <i>TMPPRSS2</i> associated with presence of variant UCB histology; CNVs in <i>miR-15a</i> , <i>CDH1</i> , <i>ZFH3</i> associated with presence of incidental prostate cancer; CNVs in <i>KLF5</i> , <i>ZFH3</i> and <i>CDH1</i> : CSS	(112)
NMIBC, MIBC, RC	39; 27	Sequencing of PT for hot spot mutations, plasma, ddPCR, personalized assays	<i>FGFR3</i> , <i>PIK3CA</i> hot spot mutations, present in PT	RFS in MIBC scheduled to RC, positive correlation between ctDNA levels from plasma and urine	(109)
Pts. with recurrent or progressive/metastatic disease, at different time points during treatment and follow up	12 (377 samples)	NGS of PT, plasma, ddPCR, 1 to 6 personalized assays, ddPCR	Genetic aberration present in PT, DEL, INS, INV, ITX, CTX for personalized assays	Follow up: up to 20 years: pts. with progressive disease had higher ctDNA levels than pts. with recurrent tumors; No ctDNA in disease-free pts.	(107)
UCB, before treatment	151, 53 controls	<i>PCDH17</i> , serum, MSP	79/151 (52.3%)	Methylation associated with higher T stage (T2-T4), G3; independent predictor of OS	(113)
UCB	10	Mutational analysis of PT, 3 digital methods	Point mutations, rearrangements	3 pts. with metastatic UCB had ctDNA	(114)
UCB	NMIBC: 75; MIBC: 20; TURBT without UCB: 48; Benign disease: 31; Healthy controls: 53	MSP, serum, digested with Bsh1236I, HpaII, HinP1I to quantify amount of methylated DNA fragments	<i>TIMP3</i> , <i>APC</i> , <i>RARB</i> , <i>TIG1</i> , <i>GSTP1</i> , <i>p14</i> , <i>p16</i> , <i>PTGS2</i> , <i>RASSF1A</i>	<10% methylated DNA, methylation levels at each site and number of methylated genes increased in UCB compared to healthy individuals, methylated DNA not correlated to stage and grade of tumors	(115)
UCB and controls	95 with UCB; 132 without UCB	Real time PCR, serum Short <i>ACTB-106</i> and large <i>ACTB-384</i> fragments; <i>ACTB-106/ACTB-384</i> : DNA integrity	UCB pts. increased <i>ACTB-106</i> levels and lower DNA integrity	No difference in <i>ACTB-106</i> levels between pts. with and without UCB, no correlation to smoking, pT stage, grade or lymph node metastasis	(116)
TCC	127 pts, 41 healthy controls	<i>CDH13</i> , serum, MSP	39/127 (30.7%) methylation of <i>CDH13</i>	Association with advanced tumour stage, poor tumour differentiation (tumour grade), larger tumour size and tumour recurrence, independently with OS	(117)

**Table 2** (continued)



Table 2 (continued)

Patients, time point of blood collection	No. of pts.	Methods	Genomic aberrations	Association of ctDNA with tumor/patient characteristics and clinical outcome	Ref.
NMIBC, different grades	42 pts., 36 healthy controls	<i>p16</i> , <i>DAPK</i> , serum, MSP	17/42 (40.5%) methylation of <i>p16</i> , methylation of <i>DAPK</i> in 27/42 cases (64.3%); in 12 pts. (28.6%) both genes methylated	Higher frequency of <i>DAPK</i> promoter methylation (71.4%) in pts. with lower grade tumors (G1)	(118)
UCB, before RC; BPH	45 UCB; 45 BPH	Serum, real time MSP	<i>APC</i> , <i>DAPK</i> , <i>GSTP1</i> , <i>PTGS2</i> , <i>TIG1</i> , <i>Reprimo</i> Hypermethylation at <i>APC</i> <i>GSTP1</i> promoters in 59.0% of UCB cases, <i>TIG1</i> (32.0%), <i>PTGS2</i> (24.0%), <i>DAPK</i> (2.0%); BPH: also methylated <i>GSTP1</i> , but not of the other genes	Hypermethylation at <i>APC</i> , <i>GSTP1</i> or <i>TIG1</i> distinguished UCB from controls with 80% sensitivity and 93% specificity, hypermethylation <i>APC</i> with stage, <i>GSTP1</i> or <i>TIG1</i> with multifocal UCB, <i>APC</i> or <i>APC</i> or <i>TIG1</i> with surgical margin positivity; <i>APC</i> hypermethylation: increased CS mortality	(119)
UCB, before RC; BPH	45 UCB; 45 BPH	qPCR, serum, 124 bp fragment of <i>PTSG2</i> , mostly apoptotic origin; 271 bp fragment of <i>Reprimo</i> (mostly necrotic origin), AI (apoptotic index ratio 124 bp/271 fragment)	Both fragments increased in UCB pts., AI increase	DNA levels and AI not correlated with clinic-pathological parameters; increased AI correlated to high UCB-specific mortality (multivariate), independent prognostic factor	(120)
UCB	86 pts., 49 controls	<i>p16</i> MSP, serum	19/86 (22.0%) with aberrant <i>p16</i> methylation	Associated with tumor presence	(121)
UCB	27	Plasma, LOH, microsatellite markers (D17S695, D17S654, D13S310, TH2, D9S747, D9S161), <i>p53</i> and <i>K-ras</i> mutations, MSP: promoter status of <i>p14ARF</i> and <i>p16INK4a</i>	Plasma: 17/27 (63.0%) displayed the same alterations in tumor tissue and plasma; Plasma: 13/15 alterations in PT (87.0%) <i>p14</i> promoter methylation, 2/5 alterations in PT (40.0%) <i>p16</i> promoter methylation	Plasma LOH associated with DFS	(122)
Superficial UCB	31	Serum and plasma; LOH with 2 markers at 17p, <i>7P53</i> microsatellites and mutations	2/8 cases: mutation of PT found in blood; 30/31 cases with 17p microsatellite changes (in blood or urine), 52.0% concordance with PT	Mutations more frequent in higher malignant superficial tumors, risk to progress to MIBC; limited value for clinical practice	(123)

Table 2 (continued)



Table 2 (continued)

Patients, time point of blood collection	No. of pts.	Methods	Genomic aberrations	Association of ctDNA with tumor/patient characteristics and clinical outcome	Ref.
Conspicuous bladder lesions, before TURBT	58	Serum and plasma; LOH with 6 microsatellite markers	Matched microsatellite changes in PT and body fluids (serum, plasma, urine) in 23 cases	Simultaneous and multiple investigations of microsatellite markers of potential clinical relevance	(124)
UCB	18; 2 controls	Serum: LOH, microsatellite analysis; comparison of DNA from sediments with that from supernatants	Higher DNA levels in supernatants than in non-centrifuged plasma samples	Centrifugation of body fluids before DNA extraction ensures high yield and quality of extracted DNA	(125)

UCB, urothelial carcinoma of the bladder; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; PT, primary tumor; ctDNA, circulating tumor DNA; NT, neoadjuvant therapy; MPLA, multiplex ligation-dependent probe amplification; ddPCR, digital droplet polymerase chain reaction; SNV, single nucleotide variation; CNV, copy number variation; mutDNA, mutated DNA; LOH, loss of heterozygosity; MSP, methylation-sensitive/specific PCR; BPH, benign prostatic hyperplasia; CS, cancer-specific; APC, adenomatous polyposis coli; DAPK, death-associated protein kinase 1; GSTP1, glutathione S transferase P; PTGS2, prostaglandin-endoperoxide synthase 2; TIG1, Tazarotene-induced gene 1; ACTB,  $\beta$ -Actin, TP53, protein 53; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PCDH17, protocadherin-17; CDH13, H-Cadherin; DEL, deletions; INS, insertions; INV, inversions; ITX, intrachromosomal translocations; CTX, inter-chromosomal translocations; qPCR, quantitative PCR.

In two retrospective studies Birkenkamp-Demtröder *et al.* and Christensen *et al.* established personalized assays based on comprehensive NGS of the corresponding primary tumors (107,109) for ctDNA analysis in plasma samples. While the first study focused on hot spot mutations in *TP53* and *PIK3CA* and found ctDNA associated with a reduced DFS in MIBC (109), the other study developed personalized assays according to genomic aberrations in the primary tumors and absent in germline DNA uncovered by three different NGS approaches. Here, they analyzed deletions, insertions, inversions, as well as intra- and inter chromosomal translocations evident in different chromosomal regions and followed the 12 patients with a total of 377 samples for up to 20 years (107). Higher levels of ctDNA detected in plasma before progression of disease were found in patients with progressive disease to MIBC, when compared to patients with recurrent NMIBC. ctDNA was no longer detectable in patients with no proof of disease during follow up (107) suggesting that monitoring of tumor progression by serial performing of personalized assays is feasible. Unexpectedly, plasma samples of two patients with progression of disease lacked ctDNA, thus ctDNA did not reflect tumor burden and actual state of the disease (107). Whether phagocytosis or upregulated DNA degradation mechanisms are responsible for elimination of ctDNA as postulated by Heidary *et al.* for a similar finding in metastatic breast cancer (128) remains to be elucidated. Moreover, clinical validation of these results on larger patient cohorts within prospective clinical trials with defined endpoints is urgently needed.

### Summary and outlook

Assays detecting and characterizing CTCs and ctDNA are valuable tools in view of clinical tumor staging and prediction of clinical outcomes for patients with UCB. CTCs detected in patients with high-risk NMIBC and MIBC prior to surgical interventions are significantly associated with disease recurrence and inferior survival. Furthermore, there is some evidence that CTCs may help identifying patients in need of systemic chemotherapy. However, interventional or randomized studies based on CTC stratification are needed for further clarification, if this biomarker is ready for prime time in patient guidance regarding multimodal therapies.

Besides enumeration, phenotypical and molecular characterization of CTCs has gained attention to identify and validate new markers intended to get implemented in

targeted therapies. Pilot studies to validate HER2 and PD-L1 expression on CTCs have been conducted and now have to be transferred to clinical studies. Although not yet applied for UCB, genetic, epigenetic and transcriptomic approaches at single CTC level have been established and are amenable to identify new therapeutic targets and to get deeper insights into tumor cell heterogeneity. A major disadvantage of CTCs as biomarker is their low prevalence in patients with early stage UCB and also the fact that there are a considerable number of patients with advanced UCB unexpectedly lacking proof of CTCs. Although standardized and automated, the CellSearch assay does not have the ability to find CTCs that have lost epithelial cell features in the course of EMT. Hence, recent technical advances in CTC approaches established to detect also mesenchymal-like CTCs mainly in breast cancer have to be exploited also for UCB.

To date, there is a strong discussion whether CTCs or ctDNA represent the better liquid biopsy tool. Indeed, ctDNA can be easily isolated from stored plasma or serum samples and if comprehensively validated has the potential to better mirror tumor cell heterogeneity and real time reflect all tumor sub-clones and hence the genomic landscape of the tumor disease. However, costs for whole exome/genome analyses are still high and personalized assays using individual markers or gene panels are dependent on previous analyses of corresponding tumor tissues, which are only rarely available in advanced tumor stages (25).

In conclusion, for UCB large-scale prospective clinical or randomized studies with defined endpoints are urgently warranted validating the promising results of liquid biopsies from available smaller observational studies. Due to complicated accrual issues in UCB trials in the past, it may be useful to include liquid biopsies in currently designed UCB interventional studies to shed more light on these blood-based biomarkers.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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