World Journal of Urology (2019) 37:1785–1799
https://doi.org/10.1007/s00345-018-2543-9

TOPIC PAPER

The current role and future directions of circulating tumor cells and circulating tumor DNA in urothelial carcinoma of the bladder

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Received: 21 June 2018 / Accepted: 22 October 2018 / Published online: 26 October 2018

Abstract

Purpose Urothelial carcinoma of the bladder (UCB) is clinically and genetically a highly heterogeneous disease. Treatment decisions are usually based on histopathological workup and molecular diagnostics on tissue biopsies of the primary tumor or the metastatic site. Next to completely different molecular genotypes of phenotypically similar tumors, standard biopsies do not unconditionally allow real-time insight during the natural course of disease progression. Indeed, in UCB there is an imperative need of biomarkers for improving clinical staging, detecting minimal residual disease, predicting therapy response and prognosis and finally enabling patient stratification for multimodal, individualized treatment and therapy monitoring. Liquid biopsies of blood-based circulating biomarkers have evolved from bench to bedside in some cancer entities.

Methods In a narrative review we are summerizing the latest evidence on CTC and ctDNA in muscle-invasive and metastatic UCB.

Results In this review, we summarize the current status, limitations and future needs of circulating tumor cells (CTC) and cell-free circulating tumor DNA (ctDNA) in UCB. Moreover, we discuss the potential clinical application of CTC and ctDNA as prognostic markers at different UCB stages and their value for target therapy guidance.

Conclusions CTC and ctDNA are promising circulating biomarkers in UCB, but none of both has progressed from bench to bedside yet. These markers may support outcome prognostication, patient counseling follow-up monitoring, and potentially decision-making regarding chemotherapy. Further prospective clinical or randomized studies are urgently warranted.

Keywords Urothelial carcinoma · Bladder cancer · Circulating tumor cells · Circulating tumor DNA · Circulating cell-free DNA · PD-L1

Introduction

With an estimated 81,190 new diagnoses and 17,240 deaths in 2017 urothelial carcinoma of the bladder cancer (UCB) is a leading cause of cancer related mortality in the western world [1]. Radical cystectomy (RC) and lymphadenectomy with or without perioperative systemic chemotherapy is the gold standard treatment for patients with muscle invasive and a subgroup of patients with high-risk non-muscle invasive UCB, respectively [2]. For patients with metastatic UCB, systemic chemotherapy is the therapeutic fundament [3].

Despite improved staging techniques, enhanced perioperative recovery support as well as an disease-free survival benefit of 9–34% with perioperative chemotherapy, up to 50% of patients treated on curative intent develop metastasis and die from UCB due to micrometastatic disease undetectable prior to definitive therapy and/or unresponsive to modern systemic treatment, respectively [4]. Indeed, in general, UCB is considered a chemosensitive disease, and systemic platinum-based combination chemotherapy is the standard of care in the neoadjuvant, adjuvant and salvage therapy setting [5]. Despite response rates about 50% to cisplatin-based chemotherapy, a non-negligible number of patients do not benefit from perioperative or salvage therapy [6]. In addition, a significant number of patients are unfit for cisplatin-based chemotherapy, and optimal systemic treatment remains unsettled [3]. Considering this, it is of tremendous

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significance to realize that UCB is not only clinically, but especially biologically and genetically a highly heterogeneous disease [7]. Phenotypically similar tumors may harbor completely different molecular genotypes representing the individuality of each tumor and its host [8]. Still, up to today all trials on systemic treatment in UCB have been conducted in unselected patients.

Tumor cell dissemination is a crucial step in the natural history of the metastatic cascade. Circulating tumor cells (CTC) are malignant epithelial cells in the peripheral blood, representing micrometastatic disease from the primary tumor or its metastasis with the propensity of evolving clinically relevant distant metastasis and potentially mirroring overall tumor burden [9]. The preponderance of CTC can be eliminated from systemic circulation due to various endogenous defense mechanisms, but it has been proven that patients with detectable viable CTC have a significantly increased risk for disease progression and unfavorable outcomes [10].

All cells, including tumor cells, release DNA into the circulatory system. Cell-free DNA (cfDNA) forms the whole pool of cell-free DNA circulating in the bloodstream. Comparable to CTC, different sources including the primary tumor, CTC, micrometastatic deposits at distant sites (e.g., bone marrow and liver) and normal cells (e.g., hematopoietic and stromal cells) contribute to the high levels of cell-free DNA (cfDNA) in the peripheral blood of cancer patients [11]. Tumor cell-derived DNA (ctDNA) represents another encouraging blood-based biomarker in various malignancies including UCB [11, 12]. CtDNA constitutes a part of this cfDNA and is genetic and/or epigenetic altered. In addition, apoptosis and necrosis along with active cell secretion as physiological events lead to an increase in cfDNA in human blood during cancer development and progression [13]. The high somatic mutation rate in UCB indicates this malignancy being ideally suited for ctDNA analysis [14]. Although ctDNA is usually investigated in the blood, urine of UCB patients is another important source for ctDNA analyses [15].

During disease progression, some malignant cell clones proliferate and develop distinct molecular aberrations [16]. Even in the same patient, metastases of different sites may be heterogeneous regarding their molecular features [17]. Circulatory biomarkers hold the potential to unmask individual genomic, epigenetic, transcriptomic and proteomic alterations in the primary tumor, apparent metastases as well as occult micrometastasis in circulation, and thus, may explain the variable clinical course of disease [8, 18]. The concept of liquid biopsy promotes the encouraging opportunity to detect and monitor disease together with therapy response without conventional biopsies or surgical excision of metastases. Both, CTC and ctDNA, are promising real-time biomarkers for dynamic disease surveillance and response monitoring [9, 10]. This is of particular interest in the beginning era of personalized medicine with individualized targeted therapies.

In this review, we summarize and discuss the value, limitations and future needs of CTC and ctDNA analyses. We analyze the prognostic capacity and discuss fundamental implications. Moreover, we scrutinize the current status and chances of these promising circulating biomarkers in different controversial issues of UCB, including personalized indications of neoadjuvant and adjuvant chemotherapy, treatment response and implications for immune-oncologic therapies, respectively.

Methods

We performed a non-systematic Pubmed/Medline literature search to identify original articles, review articles, editorials and comments regarding CTC and ctDNA in association with UCB. Searches were limited to the English language. Keywords included urothelial cancer or carcinoma, bladder cancer, transitional cell carcinoma, circulating tumor cells, circulating tumor DNA, circulating cell-free DNA, plasma DNA, serum DNA, cystectomy, metastasis, survival and prognosis. The literature search was timely unlimited, but our article focuses on the most significant findings from the past 5 years. Articles with the highest level of evidence were selected and reviewed.

Results

CTC detection techniques and technical aspects

CTC are rare cells detectable in the peripheral blood circulation with frequencies of only about 10^{-7} to 10^{-6} among normal white blood cells. Due to the rarity of CTC, elaborate techniques are necessary for the enrichment, detection, identification and analysis of CTC. Different features of CTC are used for positive or negative enrichment and capturing of CTC among them the expression of epithelial cell antigens such as keratins, EpCAM or epidermal growth factor receptors, cell size, density, plasticity, dielectrophoretic mobility or antigen expression [19, 20]. Currently more than 50 assays are available, of which enrichment and detection methods base on physical properties [21]. Still, the CellSearch® system is the only standardized platform for CTC detection that has already been cleared by the Food and Drug Administration (FDA) for the analysis of blood from patients with metastatic breast, prostate and colorectal cancer [16, 22–24]. The blood has to be drawn into CellSave® tubes ensuring stability of CTCs at room temperature up to 96 h. This approach uses anti-EpCAM antibodies coupled to

ferrofluid to immunomagnetically enrich and fluorescently labeled anti-keratin antibodies to identify CTC. Exclusion of leukocytes occurs by anti-CD45 antibodies. Interestingly, it has been shown that 96% of bladder tumors in cystectomy specimens express EpCAM [25]. Thus, the in vitro sensitivity of CellSearch® is high reaching 80% and more for the detection of CTC in UCB [26]. Numerous studies demonstrated a high sensitivity, specificity and reproducibility of CTC detection by the CellSearch® system [16, 22, 27, 28]. Currently, the majority of published studies on CTC in UCB rely on the CellSearch® system. However, there are various other CTC detection approaches available such as microfluidic technologies, size-based or sensitive real-time PCR approaches demonstrating clinical relevance of CTC [20]. Indeed, some studies provided evidence for a prognostic relevance for the detection of epithelial or UCB-specific transcripts [16, 21]. Despite there are differences in detection rates between platforms in individual patients [29], the overall detection rates are comparable for different platforms in similar patient cohorts (i.e., non-metastatic/metastatic patients) across the literature [21].

CTC in patients treated with radical cystectomy

Table 1 presents selected studies on CTC data in UCB patients treated with RC. Using CellSearch®, most studies reported detection of CTC in 20–30% of patients prior to RC [26, 30–38], with a median CTC count of one–two per 7.5 ml blood [33, 36–38]. A positive CTC status is defined as the presence of at least one CTC per 7.5 ml blood. Using other platforms than CellSearch®, similar or higher CTC counts were observed. Although data are scarce, no intra- or postoperative release of CTC into the circulatory system was found during RC [32]. There were no associations between the presence of CTC and clinico-pathologic UCB features in the most studies [31, 33, 35]. Accordingly, detection of CTC does not predict extra-vesical tumor growth [31]. More recent studies with the highest number of included patients, however, found a positive CTC status being associated with unfavorable tumor characteristics, e.g., advanced pathological tumor stage, positive soft tissue surgical margin and lymphovascular invasion [37, 38]. Up to 25% of patients present variant UCB histologies (i.e., the combination of UCB with any other histology) in RC specimens [39]. Interestingly, in patients with the presence of variant histology, the CTC detection rate is comparable to those with pure UCB at RC [37].

Most studies demonstrate an association of the CTC presence with an increased risk of disease recurrence, cancer-specific and overall mortality following RC [31, 33, 34, 36–38]. The unfavorable impact of CTC on UCB prognosis has been confirmed in a meta-analysis on 30 studies including more than 2000 patients [40]. When adjusting for standard survival prognosticators, a positive CTC status remains a powerful, independent risk factor for inferior outcomes [33, 36–38]. In contrast to other solid malignancies, which have established a cut-off value of three–five CTC [41–43], the detection of a single CTC also has a negative impact on UCB outcomes [33, 34]. In addition, CTC represents an independent predictor for poor outcomes unattached of the histopathologic subtype [37].

Besides detection and enumeration of CTC by immunocytochemical approaches including the CellSearch system, clinical relevance of CTC in UCB was also demonstrated by applying other methods including mainly those based on the detection of epithelial- or UCB-specific mRNA by RT-PCR. Thus, promising transcripts suitable as surrogates for CTC comprise keratins, epidermal growth factor receptors or uroplakins which have been shown to have prognostic relevance for DFS, RFS, CSS or OS in different studies [16].

There is still ongoing controversy on the efficacy and best time of administration of perioperative systemic chemotherapy in UCB. Despite a proven survival benefit of 5% at 5 years, administration rates for neoadjuvant chemotherapy even in high-volume centers are rather low [44]. Among others, reasons for this reluctance are imperfect selection tools for avoidance of over- or under-treatment [44]. It was demonstrated that high CTC counts prior to neoadjuvant chemotherapy are associated with unfavorable tumor features at RC [29]. Currently, the prospective interventional CirGuidance study (NTR4120) is evaluating whether CTC may serve as a biomarker to guide treatment decision on neoadjuvant chemotherapy, and completed patient accrual in April 2018 (personal communication with Dr. J.L. Boormans). In the adjuvant chemotherapy setting, recent meta-analyses also demonstrated a survival advantage, although underlying studies had relevant methodological shortcomings [45]. In a prospective study including 185 patients, the presence of CTC was associated with inferior outcomes in patients without administration of AC, but not in patients with administration of AC [38]. The authors concluded that CTC may be useful for decision-making pro or contra adjuvant chemotherapy, particularly in patients, who otherwise would not be counseled regarding systemic treatment based on their pathologic risk factors [4, 38].

CTC in metastatic UCB

Table 2 presents selected studies on CTC in metastatic UCB treated with various therapy modalities. Overall, the detection rate and number of CTC are higher compared to non-metastatic UCB. CTC are detectable in 26–91% of patients with lymph node metastasis [33, 46] and in 33–100% viscerally metastasized patients [26, 30, 36, 46–49]. The median number of CTC varies up to 100 per 7.5 ml blood [26, 30, 46–48]. Of interest, the presence and the number of CTC

Table 1 Circulating tumor cells in patients treated with radical cystectomy for bladder cancer with or without systemic chemotherapy

Study and reference	CTC assay	Patients' age (years)	Number of patients	Follow-up	Administration of chemotherapy [number of patients (%)]	Number of patients per disease stage (%)	Positive CTC status [number of patients (%)]	CTC count per patient	Association of CTC status with clinicopathologic characteristics	Association of CTC status with survival
Karl et al. [32]	CellSearch®	66.8 (median)	5	n.s.	n.s.	pT1: 1 (20.0) pT2: 3 (60.0) pT3: 1 (20.0)	1 (20.0)	2	Positive CTC status with pT ≥ 2	n.s.
Guzzo et al. [31]	CellSearch®	67.5 (mean)	43	n.s.	Neoadjuvant chemotherapy: 16 (37)	pT < 1: 8 (18.6) pT1: 5 (11.6) pT2: 6 (14.0) pT3: 12 (27.9) pT4: 12 (27.9)	9 (21.0)	1 (median)	No	n.s.
Rink et al. [36]	CellSearch®	67 (median)	55	12 months	Adjuvant chemotherapy: 8 (14.5)	pT < 1: 6 (12.2) pT1: 9 (18.4) pT2: 10 (20.4) pT3: 15 (30.6) pT4: 9 (18.4)	15 (30.0)	1 (median)	Positive CTC status with lymphovascular invasion	Positive CTC status with reduced recurrence-free, cancer-specific and overall survival
Rink et al. [33]	CellSearch®	66 (mean)	100	16 months (median)	Adjuvant chemotherapy: 20 (20)	pT < 1: 19 (19.0) pT1: 15 (15.0) pT2: 20 (20.0) pT3: 32 (32.0) pT4: 14 (14.0)	23 (23.0)	1 (median)	No	Positive CTC status with reduced recurrence-free, cancer-specific and overall survival
Soave et al. [38]	CellSearch®	68 (median)	185	31 months (median)	Adjuvant chemotherapy: 50 (27)	pT < 1: 39 (21.1) pT1: 18 (9.7) pT2: 45 (24.3) pT3: 59 (31.9) pT4: 24 (13.0)	41 (22.2)	1 (median)	Positive CTC status with lymphovascular and microvascular invasion and positive soft tissue surgical margin	Positive CTC status with reduced recurrence-free, cancer-specific and overall survival in patients without administration of adjuvant chemotherapy

Table 1 (continued)

Study and reference	CTC assay	Patients' age (years)	Number of patients	Follow-up	Administration of chemotherapy [number of patients (%)]	Number of patients per disease stage (%)	Positive CTC status [number of patients (%)]	CTC count per patient	Association of CTC status with clinicopathologic characteristics	Association of CTC status with survival
Soave et al. [37]	CellSearch®	68 (median)	188	25 months (median)	Adjuvant chemotherapy: 52 (27.7)	pT < 1: 39 (20.8) pT1: 18 (9.6) pT2: 45 (23.9) pT3: 61 (32.4) pT4: 25 (13.3)	42 (22.3)	1 (median)	Positive CTC status with pathological tumor stage, lymphovascular invasion, positive soft tissue surgical margin and administration of adjuvant chemotherapy	Positive CTC status with reduced recurrence-free, cancer-specific survival in patients with or without the presence of variant histology
Alva et al. [29]	CellSearch® and IsoFlux™	49–82 (range)	20	n.s.	Neoadjuvant chemotherapy: 20 (100)	pT < 1: 5 (31.3) pT1: 2 (12.5) pT2: 2 (12.5) pT3: 4 (25.0) pT4: 3 (18.7)	n.s.	13 (median) prior start of neoadjuvant chemotherapy	Increasing CTC number with adverse pathological tumor stage at radical cystectomy	n.s.
Abrahamsson et al. [25]	CellSearch®	73 (median)	88	16.5 months (median)	Adjuvant chemotherapy: 5 (6.7) Neoadjuvant chemotherapy: 25 (33.3)	pT < 1: 29 (38.6) pT1: 3 (3.4) pT2: 18 (24.0) pT3: 15 (20.0) pT4: 10 (13.3)	17 (19)	3 (median)	n.s.	Positive CTC status with increased risk of disease progression
Chalfin et al. [82].	AccuCyte-CyteFinder®	71 (median)	38	n.s.	n.s.	NMIBC: 8 MIBC: 12 Metastatic UCB: 9 Healthy controls: 9	2 (25) patients with NMIBC 7 (58) with MIBC 6 (67) with metastatic UCB	NMIBC: 0 MIBC: 1 metastatic UCB: 1	n.s.	n.s.

Table 1 (continued)

Study and reference	CTC assay	Patients' age (years)	Number of patients	Follow-up	Administration of chemotherapy [number of patients (%)]	Number of patients per disease stage (%)	Positive CTC status [number of patients (%)]	CTC count per patient	Association of CTC status with clinicopathologic characteristics	Association of CTC status with survival
Anantharaman et al. [59]	EpiScience	67 (median)	25	n.s.	Neoadjuvant chemotherapy or previous chemotherapy: 15 (60)	MIBC: 4 metastatic UCB: 15	20 (80) inclusive of KRT + CTC KRT–CTC: 14 (56) KRT + CTC Clusters: 6 (24) Apoptotic CTC: 13 (52) 7 (28) PD-L1 + CTC	n.s.	n.s.	High PD-L1 + / CD45 − CTC burden and low burden of apoptotic CTC with worse overall survival
Desgrandchamps et al. [87]	Ficoll	n.s.	33	n.s.	Palliative chemotherapy in one patient	pTa–pT1: 20 pT2–pT4: 11	1 (3.2%)	n.s.	n.s.	n.s.
Soria et al. [90]	TRAP-Assay	68.5 (median)	30 UCB 17 healthy controls	n.s.	Concurrent radiotherapy and chemotherapy in nine patients; chemotherapy in five patients	T2: 13 T3: 9 T4: 7 TX: 1	27 (90) with high grade, MIBC or m UCB	n.s.	n.s.	n.s.
Pagliarulo et al. [89]	Ficoll	67 (median)	59	42	Adjuvant chemotherapy: 22 patients	T0–Ta–T1: 19 (32) T2: 13 (22) T3: 21 (35) T4: 6 (10)	23 (38.9)	n.s.	CK7 expression with T stage and lymph node status	CK7 expression with increased risk for recurrence, decreased cancer-specific survival, overall survival
Kinjo et al. [88]	Buffy coat, nested RT-PCR	67 (median)	38 with UCB; 18 non-cancer pts.	n.s.	n.s.	Ta: 18 Tis: 1 T1: 10 T2: 4 T4: 1 TxN + : 4	No MUC7 mRNA in control group, 18 (47) with UCB	n.s.	MUC7 with higher grade of differentiation	n.s.

CTC circulating tumor cells, *n.s.* not specified, *pT* pathological tumor stage

Table 2 Circulating tumor cell detection in patients with metastatic bladder cancer

Study and reference	CTC assay	Patients' age (years)	Treatment [number of patients (%)]	Follow-up	Number of patients	Number of patients per disease stage (%)	Positive CTC status [number of patients (%)]	CTC count per patient	Association of CTC status with clinico-pathologic characteristics	Association of CTC status with survival
Flaig et al. [30]	CellSearch®	61.0 (median)	Chemotherapy: 14 (31.8) Surgical: 30 (68.2)	337 days (median)	44	pT ≤ 2: 2 (4.5) pT3–4: 28 (63.6) cM+: 14 (31.8)	pT3–4: 5 (18.0) cM+: 7 (50.0)	pT3–4: 1–6 (range) cM+: 1–177 (range)	n.s.	Positive CTC status in patients with cM+: trend of association with inferior overall survival
Gallagher et al. [47]	CellSearch®	68.0 (median)	Chemotherapy: 26 (78.8)	n.s.	33	cM+: 33 (100.0)	14 (33.0)	0 (median)	Positive CTC status with cM+ and the number of metastasis	n.s.
Naoe et al. [26]	CellSearch®	n.s.	n.s.	n.s.	26	cM−: 12 (46.2) cM+: 14 (53.8)	cM−: 0 (0.0) cM+: 8 (57.1)	9,2 (mean)	Positive CTC status with cM+	n.s.
Okegawa et al. [46]	CellSearch®	cM−: 71.0 (median) cM+: 68.0 (median)	Chemotherapy: 13 (65)	n.s.	36	cM−: 16 (44.4) cM+: 20 (55.6)	cM−: 0 (0.0) cM+: 11 (55.0)	0 (median)	Positive CTC status with cM+ and the number of metastasis	n.s.
De Santis et al. [49]	CellSearch®	n.s.	Chemotherapy: 69 (100)	n.s.	69	cM+: 66 (95.7)	42 (66)	n.s.	n.s.	n.s.

cM clinical presence of metastasis, *CTC* circulating tumor cells, *n.s.* not specified, *pT* pathological tumor stage

seem to be associated with the metastatic burden [46, 47]. The presence of CTC also seems to be associated with metastatic disease on FDG-PET–CT imaging studies [25]. In congruence with non-metastatic UCB, the presence of CTC is associated with inferior survival in metastatic UCB [30]. CTC may be useful for treatment monitoring in metastatic UCB, since an increasing number of CTC is associated with disease progression following radiotherapy and RC [48].

Characterization of CTC

The feasibility of phenotypical and molecular characterization of CTC up to the single cell level has been demonstrated [50, 51]. This offers the opportunity to reveal biologic properties, such as uncovering the malignant origin of these cells or exposing crucial therapeutic targets at single CTC level. In UCB, immunocytochemical and immunohistochemical analyses of CTC and primary tumor of RC specimens and lymph node metastasis showed 64% and 100% correlation of the HER2 status, respectively [33]. In patients with metastatic UCB refractory to platinum-based chemotherapy, the HER2 status correlated between CTC and primary tumor [49]. These results suggest that a subgroup of patients with HER2-positive CTC may benefit from HER2 targeting therapy, although there is currently limited evidence regarding the efficacy of Trastuzumab, a HER2 antibody, in UCB [3]. In addition, these findings potentially may suggest the origin of CTC to the primary bladder tumor [33]. However, it has to be emphasized that HER2 is not an established prognostic marker for therapy success in UCB patients. Currently, it is not possible predicting treatment response to Trastuzumab or other HER2-targeted drugs according to the HER2 status of CTC. There is only one published study reporting an association of CTC detection and a molecular subtype of UCB. Lima et al. found that CTC and the metastasis present a basal phenotype and express the Sialyl-Tn antigen, a cancer-associated glycan antigen, two factors that are associated with aggressive tumor biology and potentially inferior outcomes [52]. Fluorescence in situ hybridization (FISH) of CTC revealed copy number variations (CNV) in 55.6% of patients with the presence of CTC, consistent with malignant transformation [30]. In other cancer entities, e.g., prostate and breast cancers, analyses of the genome and transcriptome in a single CTC have successfully been performed [53, 54]. In UCB, however, these analyses have not yet been established. This may be due to the relatively low number of CTC detected in UCB patients, which renders genomic and transcriptomic profiling rather difficult.

PD-L1 expression in CTC

In regards of CTC characterization, the determination of PD-L1 expression on CTC is currently a topic of tremendous interest. Since FDA- and EMA-clearance of PD-1/PD-L1 checkpoint inhibitor treatment for advanced and metastatic UCB [55], detection of PD-L1-positive CTC might offer the opportunity to monitor response to immune checkpoint therapies. Thus, strong efforts have been made to establish a reliable approach to measure the PD-L1 status on CTC in different tumor entities. First proof-of principle studies provided evidence on PD-L1 expression in a substantial percentage of CTC derived from mainly non-small cell lung cancer, but also from breast, prostate, colorectal as well as head and neck cancer, using different technologies and a variety of different antibodies [56]. In some studies, PD-L1 expression on CTC was measured using the fourth channel of the CellSearch system [57], while other groups applied alternative methods enabling also the detection of PD-L1 expression on mesenchymal-like CTC [58]. In general, the occurrence of PD-L1-positive CTC was associated with a worse survival in compared to cases with PD-L1-negative CTC in different malignancies.

The first study determining PD-L1 expression on CTC derived from muscle-invasive and metastatic UCB was recently published. In this study, using immunofluorescence staining for nucleated cells, a method developed by EpicScience, PD-L1 expression was demonstrated on both, keratin-positive and keratin-negative, CTC. Despite a limited number of patients ($n = 25$), patients with PD-L1-positive CTC had worse overall survival [59]. Since these results are premature, currently there is no clear evidence showing that patients with PD-L1 expressing CTC may be better candidates for immunotherapy compared to CTC-positive patients without PD-L1 expression.

Circulating tumor DNA

The clinical relevance of ctDNA in UCB is sustained by its high somatic mutation rate, whose detection may be informative for disease surveillance at different stages and different times. The size of cfDNA may indicate its source of origin. Apoptotic cells produce DNA fragments of 180–200 bp or multiples of this unit, whereas necrotic cells release higher molecular-weight DNA fragments in size of over 10,000 bp [60]. Considering its quality, the different cfDNA fragment lengths have important implications in the measurement and analysis of ctDNA. For example, as reported by Ellinger et al., the fragments of mitochondrial DNA (mtDNA) are somewhat longer in UCB patients. These researchers found that the integrity defined as ratio of mtDNA-220 to mtDNA-79 fragments was increased in serum of UCB patients compared to control subjects and prostate cancer patients [61]. Estimations indicate that a significant amount of up to 3.3% of tumor DNA is released into the bloodstream every day depending on the tumors size [11]. The amount of ctDNA in the whole pool of cfDNA containing both tumor and normal

cfDNA may significantly vary from 0.01 to 50% among cancer patients, and be related to tumor size. In MIBC patients, the ctDNA fraction may even increase above 50% of cfDNA. In contrast, average cfDNA yields in MIBC before therapy seem to be less than 10ng per ml of plasma (representing only 1500 diploid genomes) [62]. Thus, the levels of cfDNA that correlate with changes in tumor burden have a great dynamic range, even greater than CTC. In human blood, this cfDNA circulates predominantly as nucleosomes [63] whose histone modifications may also be tumor-specific [64]. From blood, cfDNA is removed by the liver and kidney, and its half-life is variable ranging from 15 min to several hours [11].

Technical aspects of ctDNA analyses

Due to the low prevalence of ctDNA in total cfDNA pool, ctDNA analyses are challenging and require sensitive detection techniques. Conventional PCR-based technologies, such as amplification refractory mutation system (ARMS-PCR) and bidirectional pyrophosphorolysis-activated polymerization (bi-PAP), are easy, rapid, cost effective and mostly used. However, their limitation is that they only analyze a limited number of genomic loci. Prominent digital PCR (dPCR)-based methods, including droplet digital PCR (ddPCR) and BEAMing (beads, emulsion, amplification and magnetics), have improved detection rates compared to conventional PCR-based methods. For example, droplet dPCR (ddPCR), frequently applied in bladder cancer, uses fluorescently labeled TaqMan probes to identify individual mutated DNA molecules that are dispersed DNA in thousands of droplets. Drawback of this technique is the time-consuming and difficult process to produce droplets. Significant progress in ctDNA detection was made by the introduction of whole genome/exome sequencing of ctDNA and has led to the detection of various novel cancer-associated genomic alterations, including single nucleotide variants (SNV), copy number alterations (CNAs) and structural changes of DNA. However, sequencing-based approaches require experience in the technique and statistics and are expensive [91].

Sources for ctDNA analyses

In UCB, cfDNA was initially analyzed in urine [65]. Although urine, particularly from UCB patients, is well eligible for cfDNA analyses, the fragmentation of cfDNA may be higher in urine than in plasma or serum, and therefore, disturb the analyses. Extensive research on ctDNA in plasma and serum of UCB started at the beginning of this century. At this time, the studies by Knobloch et al. [66] and Utting et al. [67] showed that microsatellite instability (MSI) assessed by fluorescence PCR cannot only be detected in cfDNA isolated from urine but also from serum and plasma of UCB patients. Simultaneously, Dahse et al. evaluated TP53 alterations as a potential marker for a non-invasive diagnosis of recurrences or residuals in superficial UCB patients, but they only re-detected TP53 mutations from the primary tumor in 25% of plasma/serum samples using direct genomic sequencing [68]. Apparently, the former sequencing method was not enough sensitive. In the same year, Domínguez et al. reported that p14ARF promoter hypermethylation or MSI in plasma was associated with recurrence in UCB patients [69]. In particular, further small studies revealed hypermethylation of APC, GSTP1, TIG1, DAPK, p16 and cadherin promoters in serum cfDNA and its association with clinico-pathologic features [70–74]. Copy number variations (CNV) of tumor suppressor genes and oncogenes are also an informative factor for UCB [12].

Although these studies are promising, well-designed systematic and large studies on cfDNA of UCB patients are urgently needed to translate its applicability to clinical practice. To develop sensitive and reliable ctDNA-based assays and a systematic demonstration of its clinical utility as tumor biomarkers, in particular, preanalytical factors of ctDNA analyses should be standardized. Preanalytical factors include selection of the matrix (plasma, serum or urine), sample collection and preparation. Usually plasma is preferred to serum, because leukocytes can be lysed in serum during coagulation/fibrinolysis. Storage temperature and time may also affect cfDNA concentration. To avoid false data, blood samples should be checked for hemolysis by hemoglobin measurements.

Circulating tumor DNA in muscle invasive bladder cancer

In MIBC, increased levels of somatic variants of ctDNA [15] as well as an increased apoptosis index as surrogate for DNA fragmentation [75] can be detected. The apoptosis index was also an independent predictor for cancer-specific mortality [75]. High levels of ctDNA have been described in some studies with similar alterations between ctDNA and tumor tissue [76].

Detection of ctDNA may also serve as a tool for the early examination of disease recurrence in MIBC patients after treatment on curative intent, in turn enabling administration of early systemic therapy before metastatic disease gets apparent by radiologic imaging [65]. In this regard, some analyses focused on hot spot mutations in FGFR3 and PIK3CA, and found that high levels of ctDNA in plasma samples were associated with recurrence in MIBC [77]. Other investigators measured single nucleotide variants by tagged amplicon sequencing and copy number alterations by shallow whole genome sequencing in plasma of MIBC patients who received neoadjuvant chemotherapy. However, they found that these aberrations were more seldom

detectable in plasma compared to the primary tumor, and did not correlate with response to neoadjuvant chemotherapy [78].

Establishing a rapid profiling method and using multiplex ligation-dependent probe amplification, our group detected that copy number variations (CNV) of some tumor suppressor and oncogenes (e.g., KLF5, ZFHX3 and CDH1) in serum cfDNA are associated with unfavorable cancer-specific survival in UCB patients treated with RC [12]. In another analysis, we found that CNV rates vary compared to the CTC status in the same cohort of patients. In our analyses both, CTC and CNV status, influenced outcomes. Thus, a combination of both investigations may mirror the most robust insight on an individual patient's circulating marker status [79].

Circulating tumor DNA in metastatic bladder cancer

As recently demonstrated by Birkenkamp-Demtröder et al., the majority of ctDNA in UCB patients seems to be shed into the blood circulation by metastatic lesions. Patients with metastatic relapse had significantly higher levels of ctDNA compared with disease-free patients. The median positive lead time between ctDNA detection in plasma and diagnosis of relapse was 101 days after cystectomy [80]. Applying a combination of whole exome sequencing and targeted sequencing, Vandekerkhove et al. revealed that 95% of metastatic UCB patients harbored deleterious alterations of TP53, RB1 or MDM2, and 70% harbored a mutation or disrupting rearrangement affecting chromatin modifiers in their plasma [62]. A very recent study demonstrated that among patients with metastatic UCB, for whom no tumor tissue was available, cfDNA next generation sequencing was able to identify a similar profile of genomic alterations for biomarker-driven clinical trials compared with tumor tissue [81]. Thus, ctDNA may reveal information on treatment relevant mutations, e.g., FGFR3, HER2, PD-L1 mutations, although currently, these markers have not been established as predictors of treatment response in ctDNA.

Discussion

Relevant numbers of CTC and ctDNA are found in the systemic circulation of patients with UCB. Despite the multiplicity of platforms for the complex enrichment and detection technologies, there is reliable evidence that the presence of these circulating biomarkers may indicate occult metastatic disease. This is of particular interest in patients with no evidence of metastatic disease on radiologic imaging, which is known for its spatial resolution limitations [18]. Circulating biomarkers seem to be prognostic biomarkers for UCB outcomes. The overall body of evidence is larger for CTC in UCB. A recent meta-analysis based on over 30 studies with over 2000 patients included found a significant association of the CTC presence with inferior disease-free, cancer-specific and overall survival, respectively [40]. In consequence, CTC may serve as a prognostic marker for patient counseling regarding outcomes, but more importantly may be used for treatment decision-making regarding neoadjuvant or adjuvant chemotherapy. In addition, particularly CTC seems to be an interesting source for disease monitoring during follow-up or therapy as they potentially may determine response to chemotherapy and suggest the existence of chemoresistant subpopulations [21]. Nevertheless, all studies were non-interventional pro- or retrospective investigations, and no randomized study confirmed the capacity of these promising biomarkers. Therefore, integration of CTC in future clinical trials is urgently warranted elucidating the true value in non-metastatic and metastatic UCB.

Interestingly, ctDNA concentrations generally show a higher dynamic range compared to the presence and numbers of CTC in UCB patients, although these higher ctDNA detection rates do not necessarily represent a larger viable tumor burden. Hence, it is important to note that a majority of CTC studies in UCB relies on the CellSearch® system. This system implicates critical limitations: it is possible that this assay fails to detect cells that lost EpCAM and/or CK expression, which frequently occurs during epithelial–mesenchymal transition (EMT). In UCB and other solid malignancies, EMT is essential for the metastatic process [9, 10]. Thus, CellSearch® potentially misses the most dedifferentiated and—from an oncological perspective—most interesting cells [4]. In consequence, assays allowing CTC capturing independent of EpCAM expression may be advantageous [82]. In addition, it remains a point of continuing discussions, whether all selected cells exactly fulfilling the morphological criteria are indeed viable CTC and vanguard of metastasis [83].

Considering ctDNA investigations, complex physiological as well as cancer induced/related mechanisms influence ctDNA analysis. For example, it is assumed that necrosis accompanies cancer development, whereas phagocytosis and apoptosis are defense responses of the organism resulting in the destruction of cancer cells as well as the surrounding non-cancer tissues [84]. In consequence, results of ctDNA analyses usually do not come to a "ctDNA present vs. not present"-conclusion, but rather report more compound variations in oncologic target or tumor suppressor genes. Depending on quality and quantity, ctDNA is an ideal source for detection of genetic and epigenetic alterations that may reflect the characteristics of the tumor and its environment including micrometastatic cells, as well as the stage of disease [85]. Thus, ctDNA immediately allows reflection of the current state of disease, and offers an elegant opportunity

of real-time serial surveillance for disease progression and response on a molecular basis.

To date, there is ongoing discussion, if CTC or ctDNA represent the better circulating biomarker. Indeed, an advantage of ctDNA is that it can easily be isolated from stored plasma or serum samples, and potentially may better reflect the molecular character of tumor cells at any time and stage during the natural course of disease. However, costs for whole exome/genome analyses are still enormous, and personalized assays using individual markers or gene panels are dependent on previous detection analyses of the corresponding tumor tissues, which are only rarely available in advanced tumor stages [16]. Indeed, ctDNA represents mainly the genome of dying tumor cells, but viable tumor cells drive disease progression and impact therapy variability [19]. The strength of CTC analyses is based on the prognostic impact of even a single cancer cell on outcomes, and the possibility of subsequent (whole-)genome and/or target-lesion analysis. Phenotypical and molecular characterization of CTC in UCB has already been established [16], including analyses of important surface markers for targeted therapy, e.g., HER2 [33] and PD-L1 status [59], among others. To our knowledge, so far there is only one preliminary study that analyzed the prognostic capacity of CTC and ctDNA in the same UCB patient population [79]. Nevertheless, CTC and ctDNA do not indiscriminately reflect the same aspects of disease, and thus, should be seen as mutual adjuncts rather than competing circulating markers.

In the evolving era of personalized, tailored medicine, there is an urgent need for robust non-invasive biomarkers to optimize patient counseling and treatment individualization [8]. Especially analyses of checkpoint inhibitory proteins on circulating biomarkers seem of tremendous future interest, as recently the FDA and EMA cleared several drugs for treatment of advanced and metastatic UCB [55]. Despite effusive initial excitement, the more data are accumulating, the more it becomes evident that although efficacy and safety of checkpoint immunotherapy are superior compared to standard chemotherapy, only about 20% of UCB patients respond [86]. Indeed, the expression pattern of any immunotherapy biomarker on tumor or immune-infiltrative cells does not possess adequate predictive value for treatment guidance [7]. The heterogeneity between the primary tumor, lymph node or distant metastasis, as well as tumor cells in the peripheral circulation are likely being an important reason for this dilemma. In addition, adaptive regulation of target protein expression during treatment may influence therapy response. A biopsy of each single primary and metastatic lesion would be needed to delineate a thorough landscape of a patients' molecular diversity [7]. In consequence, circulating biomarkers represent promising tools mirroring the intra-individual genetic and epigenetic heterogeneity. Serial liquid biopsies may be an elegant solution for standardized real-time monitoring of early assessment of therapy effects and potentially response.

A limitation of our current review is that we did not quote all platforms and techniques for circulating biomarker analysis, as we focused on papers with the highest evidence for this summary. For CTC and ctDNA analyses, a myriad of commercially available technological platforms with different strength and weaknesses exist [21]. While diversity aims at improving the findings, the plurality of technical developments also has slowed down the introduction of circulating biomarkers into clinical diagnostics [19]. From clinical perspective, standardized approaches with a high sensitivity and specificity as well as valid reproducibility allowing comparability between different systems are of major importance.

Conclusion

Fundamental research over the last decades has provided vital insight into the molecular pathogenesis and complex signaling pathways of UCB. CTC and ctDNA are promising circulating biomarker, which paved the road for liquid biopsy diagnostics in cancer, including UCB, although none of both has progressed from bench to bedside. Evidence from the available literature justifies the conclusion that circulating biomarkers in UCB are relevant outcome prognosticators and maybe beneficial in patient counseling regarding follow-up monitoring, decision-making regarding chemotherapy and potentially individualized treatments. Prospective clinical or randomized studies are urgently warranted validating the promising results of liquid biopsies in UCB from available observational studies. It may be useful including liquid biopsies in currently planned UCB trials to shed more light on circulating biomarkers.

Author contributions MR: project development, data analysis, manuscript writing, and manuscript editing. HS: data collection, manuscript writing, and manuscript editing. SR: data collection, manuscript writing, and manuscript editing. AS: data collection, manuscript writing, and manuscript editing.

Compliance with ethical standards

Conflict of interest Dr. Michael Rink received honoraria by BMS and Pfizer. Dr. Michael Rink is investigator for the following companies and receives research grants by the following companies: Pfizer, MSD. All other authors have nothing to disclose.

Informed consent All authors consented to the final draft.

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