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REVIEW ARTICLE

Circulating tumor cells as promising novel biomarkers in solid cancers*

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Abstract

The presence of circulating tumor cells (CTCs) in peripheral blood can serve as a ''liquid biopsy'' approach and has thus emerged lately as one of the hottest fields in cancer research. CTCs can be isolated from blood in a non-invasive approach, and can be used to follow patients over time since these cells can provide significant information for a better understanding of tumor biology and tumor cell dissemination. CTC molecular characterization offers the unique potential to better understand the biology of metastasis and resistance to established therapies, and analysis of these cells presents a promising field for both advanced and early-stage patients. CTC detection, enumeration, and molecular characterization are very challenging since CTCs are rare, and the amount of available sample is very limited. Since detection of CTCs has been shown to be of considerable utility in the clinical management of patients with solid cancers, various analytical systems for their isolation and detection have been developed. New areas of research are directed towards developing novel assays for single-CTC isolation and molecular characterization. The clinical significance of CTCs has been evaluated in many types of solid cancers, and the CTC enumeration test in metastatic breast, colorectal, and prostate cancer was cleared by the FDA almost a decade ago. This review is mainly focused on the clinical potential of CTCs as novel biomarkers in 10 different types of solid cancers: breast, ovarian, prostate, lung, colorectal, hepatocellular carcinoma, pancreatic, head and neck, bladder cancer and melanoma.

Keywords

Circulating tumor cells, CTC, liquid biopsy, molecular characterization, prognostic biomarkers, predictive biomarkers, solid cancers

History

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Abbreviation: **AA**: abiraterone acetate; **AR**: androgen receptor; **CRPC**: castration-resistant prostate cancer; **cfDNA**: cell-free DNA; **CTCs**: circulating tumor cells; **CK-19**: cytokeratin-19; **CK-7**: cytokeratin-7; **DFS**: disease-free survival; **EGFR**: epidermal growth factor receptor; **EMT**: epithelial–mesenchymal transition; **HCC**: hepatocellular carcinoma; **hTERT**: human telomerase reverse transcriptase; **LAPC**: locally advanced pancreatic carcinoma; **LAHNC**: locally advanced head and neck cancer; **mCRC**: metastatic colorectal cancer; **NSCLC**: non-small cell lung cancer; **OS**: overall survival; **PFS**: progression-free survival; **PSA**: prostate-specific antigen; **RT-PCR**: reverse transcriptase-polymerase chain reaction; **SNUC**: sinonasal undifferentiated carcinoma; **SCCHN**: squamous cell carcinoma of the head and neck; **TTF-1**: thyroid transcription factor 1; **TGF-β**: transforming growth factor-beta; **TMPRSS2**: transmembrane protease, serine 2

Introduction

The presence of tumor cells circulating in the blood of cancer patients was first reported by Thomas Ashworth in 1869[1]. Almost 150 years after this first report, the importance of circulating tumor cells (CTCs) detection and molecular characterization is becoming evident[2]. CTCs can be isolated in a non-invasive way, and can thus be used as a ''liquid biopsy'' to follow patients over time. These cells can provide significant information for a better understanding of tumor biology and tumor cell dissemination. Their molecular characterization offers the unique potential to better understand the biology of metastasis and resistance to established therapies[3,4]. CTC analysis presents a promising field for both advanced and early-stage cancer patients.

CTC detection, enumeration, and molecular characterization are extremely challenging since CTCs are very rare, and the amount of available sample is quite limited. Since detection of CTCs has been shown to be of considerable utility in the clinical management of patients with solid cancers, various analytical systems for their isolation and detection have been developed[5–8]. New areas of research are directed towards developing novel assays for CTC molecular characterization[6,9,10]. High heterogeneity of CTCs, even among the same individuals, has been observed when performing high-dimensional single CTC profiling, and by

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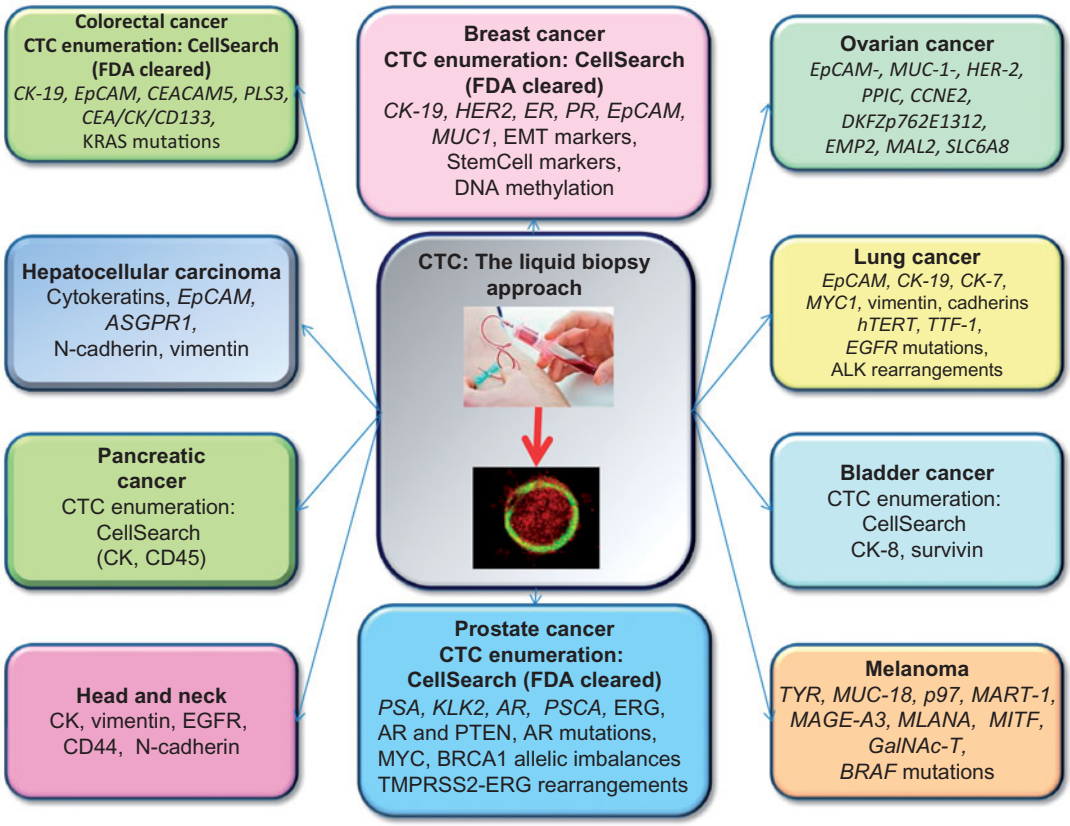

Figure 1. CTC analysis and molecular characterization in various types of solid cancers.

directly measuring gene expression in individual CTCs without the common practice of pooling such cells[9]. However, many questions still remain unanswered regarding the biology of CTCs, the optimal method to enumerate and characterize them, and the path to regulatory and general clinical acceptance of technology platforms currently under development[7].

The aims of research on the clinical potential of CTCs include (a) estimation of the risk for metastatic relapse or metastatic progression, (b) patient stratification and real-time monitoring of treatment efficacy, (c) identification of therapeutic targets and resistance mechanisms, and (d) understanding metastasis development in cancer patients[11].

This review is mainly focused on the clinical potential of CTCs as novel biomarkers in 10 different types of solid cancers: breast, ovarian, prostate, lung, colorectal, hepatocellular carcinoma, pancreatic, head and neck, bladder cancer, and melanoma (Figure 1).

Breast cancer

The first comprehensive meta-analysis of the published literature on the prognostic relevance of CTCs in patients with early-stage and metastatic breast cancer clearly indicated that CTC detection is a reliable prognostic factor[12].

Metastatic breast cancer

In their seminal paper many years ago, Cristofanilli and colleagues clearly showed, using the CellSearch System (Veridex, South Raritan, NJ), that CTCs represent an independent prognostic factor for progression-free survival (PFS) and overall survival (OS) in patients with metastatic breast cancer, and that a cut-off of 5 CTCs/7.5 mL of blood in these patients was highly predictive of clinical outcome[13]. This paper revolutionized the clinical applications of CTCs in many types of cancer since it led to the FDA clearance of the CellSearch assay that is standardized, semi-automated, and not subject to pre-analytical errors. Since then, a plethora of clinical studies has verified the importance of CTC enumeration in metastatic breast cancer[14–18].

CTC clearance could possibly be used as a ''surrogate'' marker for potentially improved survival for regulatory purposes. Many ongoing clinical studies, based on different designs in various patient populations, are now evaluating the potential of CTC implementation in the routine management of breast cancer patients[19].

Early breast cancer

The prognostic value of CTCs in axillary lymph node-negative breast cancer patients, based on a nested RT-PCR, was already shown in 2002[20]. By using a real-time RT-qPCR assay for *CK-19* mRNA[21,22], CTC detection was shown to be an independent prognostic factor for reduced disease-free interval and overall survival before[23], during[24], and after[25] chemotherapy in early breast cancer. Detection of CTCs before adjuvant chemotherapy predicted for poor clinical outcome mainly in patients with ER-negative, triple-negative, and HER2-positive early-stage breast cancer[26]. When CTCs were prospectively detected before and after neoadjuvant chemotherapy in a phase II trial, it was found that detection of one or more CTCs in 7.5 mL of blood before neoadjuvant

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chemotherapy can accurately predict OS[27]. A more recent study investigated the value of CTC detection during the first five years of follow-up in predicting late-disease relapse. It showed that persistent detection of CTCs was associated with an increased risk of late-disease relapse and death in patients with operable breast cancer and indicated the presence of chemotherapy- and hormonotherapy-resistant residual disease[28]. Lucci et al. prospectively collected data on CTCs at the time of definitive surgery from chemonaïve patients with stage 1–3 breast cancer. They enumerated CTCs and assessed outcomes at a median follow-up of 35 months, and showed that the presence of one or more CTCs predicted for early recurrence and decreased overall survival in chemonaïve patients with non-metastatic breast cancer[29].

Molecular characterization of CTCs in breast cancer as surrogate markers for treatment response

There is now a growing body of evidence that the hormone receptor and HER2 status in CTCs can be different from that in the primary tumors and even change over time, especially during disease recurrence or progression in breast cancer patients[30–35]. Based on this evidence, re-evaluation of hormone receptor and HER2 status by molecular characterization of CTCs is a strategy with potential clinical application. However, based on the current guidelines, hormone therapy and anti-HER2 therapy are prescribed according to the hormone receptor (ER/PR expression) and HER2 status of the primary tumor. An optimal, individualized treatment could be selected by characterizing ER and HER2 status in CTCs and comparing it with the primary tumor[36]. Ligthart et al. have recently developed an automated algorithm for evaluating HER2 expression in CTCs when using the CellSearch system. They report that the HER2 expression is very heterogeneous among CTCs within each patient[37]. Many research groups have already shown that HER2-positive CTCs can be detected in patients with HER2-negative primary tumors[30,32,34,38,39]. Georgoulias et al. were the first to investigate the effect of trastuzumab in HER2(−) patients that have CK(+)/HER2(+) CTCs in a randomized phase II study. According to their results, administration of trastuzumab can eliminate chemotherapy-resistant *CK-19* mRNA-positive CTCs, reduce the risk of disease recurrence, and prolong disease-free survival (DFS)[40].

In non-metastatic breast cancer patients, the expression of estrogen, progesterone, and epidermal growth factor (EGF) receptors as detected by immunofluorescence experiments revealed heterogeneous expression of these hormonal receptors in samples from the same patients[41].

CTCs play a crucial role in metastasis, and epithelial–mesenchymal transition (EMT) is an essential process in the metastatic cascade[42,43]. A major proportion of CTCs of metastatic breast cancer patients show EMT and tumor stem cell characteristics[44]. As well, CTCs expressing Twist and vimentin were identified in patients with metastatic and early breast cancer[45]. The existence of a subpopulation of CTCs with putative stem cell/progenitor phenotypes in patients with metastatic breast cancer has been shown by using triple-marker immunofluorescence microscopy[46]. Currently used detection methods for CTCs are not sufficient at identifying this subtype of CTCs that underwent EMT[47].

Very recently, Yu et al. showed by serial monitoring of CTCs in patients with breast cancer that these cells simultaneously expressed mesenchymal and epithelial markers. They showed that mesenchymal cells expressing known EMT regulators, including transforming growth factor (TGF)-β pathway components and the FOXC1 transcription factor, were associated with disease progression[48]. The expression levels of EMT-inducing transcription factors have been determined in CTCs in primary breast cancer patients[49]. Investigation of the apoptotic and proliferative status in CTCs of breast cancer patients has shown that patients with metastatic and advanced disease had significantly lower numbers of apoptotic CTCs compared with patients with early breast cancer. In addition, adjuvant chemotherapy reduced both the number of CTCs per patient and the number of proliferating CTCs[50].

Molecular characterization of isolated CTCs at the DNA methylation level has shown that tumor suppression is severely disabled in CTCs via progressive DNA hypermethylation. This modification leads to epigenetic silencing of key tumor suppressors and metastasis suppressors known to affect hallmark properties of tumor cells, including growth and proliferation, invasiveness, epithelial phenotype and stemness. *CST6, BRMS1*, and *SOX17* genes were found to be methylated in CTCs[51]. The *SOX17* promoter was highly methylated in primary breast tumors, in CTCs isolated from both early and metastasis-verified breast cancer patients, and in corresponding cell-free DNA (cfDNA) samples[52]. A key finding from these studies is that for the first time, *SOX17* promoter methylation in CTCs and in matched cfDNA was shown to be highly correlated. This finding indicates a direct connection between the presence of CTCs and cfDNA in operable breast cancer patients after surgical removal of the primary tumor.

Tables 1 and 2 list CTCs in early and metastatic breast cancer and their molecular characterization and clinical significance.

Ovarian cancer

Obermayr et al. identified a panel of six genes for the PCR-based detection of CTCs in female cancer patients. They reported that by using this panel, they could identify 44% of the cervical, 64% of the endometrial, and 19% of the ovarian cancer patients[53]. The same group, in a more recent study, identified novel markers for CTCs in patients with epithelial ovarian cancer and evaluated their impact on clinical outcome. By using these markers, they could detect CTCs in 24.5% of the baseline (before primary treatment) and 20.4% of the follow-up samples (six months after adjuvant chemotherapy), of which two-thirds were identified by overexpression of the cyclophilin C gene (PPIC), and just a few by *EpCAM* overexpression. They report that the presence of CTCs at baseline correlated with the presence of ascites, suboptimal debulking, and elevated CA-125 and HE-4 levels, whereas CTCs during follow-up occurred more often in older and platinum-resistant patients. PPIC-positive CTCs during follow-up were detected significantly more often in the

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Table 1. Molecular characterization and clinical significance of CTCs in early breast cancer.

Detection method used	Biomarker used	Prognostic significance	References
RT-qPCR	*CK-19*	Not evaluated	21–22
RT-qPCR	*CK-19*	OS: yes DFS: yes	23–26,28
RT-PCR, IF	*CK-19 , HER2*	DFS: yes	40
CellSearch	Enumeration of CTCs	OS: yes DFS: yes	29
CellSearch	*HER2*	Not evaluated	32
IF, FISH	ER, PR, EGFR, HER2, and TOP2A	Not evaluated	41
AdnaTest BreastCancer, Multiplex RT-PCR	*EpCAM, MUC-1* and *HER2, Twist1, Akt2, PI3K, ALDH1*	Not evaluated	47
IF	TWIST1, SNAIL1, SLUG, ZEB1, and FOXC2	Not evaluated	48
IF	CK+, M30, Ki67	Not evaluated	50
Methylation-specific PCR	*CST6* promoter methylation, *BRMS1* promoter methylation, *SOX17* promoter methylation	Not evaluated	51

Table 2. Molecular characterization and clinical significance of CTCs in metastatic breast cancer.

Detection method used	Biomarker used	Prognostic significance	References
CellSearch	Enumeration of CTCs	OS: yes PFS: yes	13–15,17
CellSearch	Enumeration of CTCs	OS: yes PFS: no	18
CellSearch	Enumeration of CTCs	OS: not evaluated PFS: yes	27
CellSearch, AdnaTest BreastCancer	*HER2*	Not evaluated	30
CellSearch, RT-PCR	55 mRNAs and 10 miRNAs	Not evaluated	33
CellSearch, IF, FISH	*HER2*	Not evaluated	34
CellSearch, IF, FISH	*HER2*	Not evaluated	39
IF	CK, Twist and vimentin	Not evaluated	45
AdnaTest BreastCancer, Multiplex RT-PCR	*EpCAM, MUC-1* and *HER2, Twist1, AKT2, PI3K, ALDH1*	Not evaluated	44
IF	*CD44(+)/CD24(-/*low*)* and *ALDH1(+)*	Not evaluated	46

Table 3. Molecular characterization and clinical significance of CTCs in ovarian cancer.

Type of cancer	Detection method used	Biomarker used	Prognostic significance	References
Primary ovarian cancer	RT-qPCR	*CCNE2, DKFZp762E1312, EMP2, MAL2, PPIC* and *SLC6A8*	Not evaluated	53
Epithelial ovarian cancer	RT-qPCR	*PPIC, EpCAM*	OS: yes DFS: yes	54
Primary ovarian cancer	AdnaTest, RT-qPCR	*EpCAM-, MUC-1-*, and *HER2, CA 125*	OS: yes	55
Advanced ovarian cancer	CellSearch	Enumeration of CTCs	OS: yes PFS: yes	56
Newly diagnosed or recurrent epithelial ovarian cancer	CellSearch	Enumeration of CTCs	OS: no DFS: no	57

platinum-resistant than in the platinum-sensitive patient group, and indicated poor outcome independent of classical prognostic parameters[54].

By using the commercially available AdnaTest BreastCancer kit (Allere, Coconut Creek, FL), based on immunomagnetic enrichment and multiplex RT-PCR for selection and detection of CTCs, Aktas et al. checked for CTCs in the blood of 122 ovarian cancer patients at primary diagnosis and/or after platinum-based chemotherapy. They reported that CTC-positivity significantly correlated with shorter OS before surgery (p=0.0054) and after chemotherapy (p=0.047)[55]. Poveda et al. evaluated the correlation between numbers of CTCs and PFS and OS in a phase III study of pegylated liposomal doxorubicin (PLD) with trabectedin versus PLD for relapsed ovarian cancer by using the CellSearch system and reagents (Veridex, South Raritan, NJ). Results from this study indicated that elevated numbers of CTCs impart an unfavorable prognosis for ovarian cancer patients[56]. Recently, Liu et al. investigated whether CTCs, as detected and enumerated by the Veridex CellSearch system, could predict for clinical outcomes in women with newly diagnosed or recurrent epithelial ovarian cancer. According to their results, CTCs can be isolated from women with newly diagnosed or recurrent ovarian cancer; however, their numbers do not significantly correlate with clinical characteristics or patient outcomes[57]. Table 3 lists CTCs in ovarian cancer and their molecular characterization and clinical significance.

Prostate cancer

In patients with advanced prostate cancer, CTC enumeration using the Veridex CellSearch system at baseline and post-treatment has been cleared by the FDA for quantifying the load of tumor cell dissemination. This test is prognostic of survival and is currently being implemented into routine clinical practice for estimating prognosis and monitoring treatment success[58]. The clinical utility of monitoring CTC changes with treatment, as an efficacy-response surrogate biomarker of survival, is currently being tested in large phase III trials with the novel anti-androgen therapies abiraterone acetate and MDV3100. Molecular determinants can be identified and characterized in CTCs as potential predictive biomarkers of tumor sensitivity to a therapeutic modality[59].

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Table 4. Molecular characterization and clinical significance of CTCs in prostate cancer.

Type of cancer	Detection method used	Biomarker used	Prognostic significance	References
Early prostate cancer	CellSearch	Enumeration of CTCs	Not evaluated	69
	FISH	*BRCA1* allelic imbalances	Not evaluated	77
Castration-resistant prostate cancer	CellSearch, FISH	*ERG, AR* and *PTEN*	Not evaluated	76
	CellSearch	Enumeration of CTCs	OS: yes DFS: not evaluated	79–80
	RT-PCR	*TMPRSS2-ERG* rearrangements	OS: yes DFS: yes	75
	Direct sequencing	*AR* mutations	Not evaluated	74
Localized-disease and metastatic CRPC	RT-qPCR	*KLK3, KLK2*, and *PSCA*	OS: yes	65
Metastatic castration-resistant prostate cancer	CellSearch	Enumeration of CTCs	OS: yes	61
	CellSearch	Enumeration of CTCs	OS: yes	62–64
	CellSearch, FISH	*AR, MYC*	Not evaluated	72
	Flow cytometry	CK, CD45	PFS: yes	60
Metastatic hormone-sensitive prostate cancer	CellSearch	Enumeration of CTCs	OS: yes PFS: yes	66

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Metastatic prostate cancer

Moreno et al. reported in 2001 that CTC levels can be quantified in the circulation of patients with metastatic prostate cancer, and that the change in the numbers of CTCs correlates with disease progression with no diurnal variations[60]. In 2007, Danila and colleagues reported that the number of CTCs before therapy was predictive of survival, with no threshold effect, in patients with castrate-metastatic disease considered for different hormonal and cytotoxic therapies. Moreover, the shedding of cells into the circulation represents an intrinsic property of the tumor, distinct from the extent of disease, and provides unique information relative to prognosis[61]. In 2008, data presented by de Bono and colleagues showed that CTC enumeration using the CellSearch system had prognostic and predictive value in patients with metastatic castration-resistant prostate cancer (CRPC) and was an independent predictor of OS in CRPC, opening the way for FDA clearance of this assay for the evaluation of CRPC[62]. CTC numbers, analyzed as a continuous variable, predict OS and provide independent prognostic information about time-to-disease progression and can be used to monitor disease status[63,64].

Real-time RT-qPCR assays for *KLK* mRNAs have been also used for the detection of CTCs in prostate cancer. It has been reported that *KLK2/3*-expressing CTCs are common in men with CRPC and bone metastases, but are rare in patients with metastases diagnosed only in soft tissues and patients with localized cancer[65]. Resel and colleagues analyzed the correlation between CTC levels and prostate-specific antigen (PSA) levels, Gleason score, and TNM stage in patients with metastatic hormone-sensitive prostate cancer. They reported that the CTC count in peripheral blood could provide a method for correctly staging prostate cancer and for assessing the prognosis of metastatic hormone-sensitive prostate cancer[66]. The combination of CTC and PSA velocity may offer insights into the prognosis and management of advanced prostate cancer[67].

Early-stage prostate cancer

Current technology cannot differentiate local from distant recurrent prostate cancer. However, up to 30% of prostate cancer patients will experience a rise in PSA, requiring radiation therapy within 10 years of radical prostatectomy. CTCs have been detected in prostate cancer and may be a new surrogate candidate towards deciding whether or not to offer systemic or local treatment[68]. CTC tests may assist with clinical decision-making according to a pilot study that investigated whether CTCs could be detected in early-stage prostate cancer patients receiving salvage radiotherapy using the CellSearch system. The results of this study demonstrated that CTCs can be detected in early-stage prostate cancer and suggest the possibility that post-treatment reduction in CTC levels may be indicative of radiation therapy response[69]. Recent trials in patients with CRPC are incorporating the detection of CTCs, imaging, and patient-reported outcome biomarkers in order to improve future drug development and patient management[70]. Table 4 lists CTCs in prostate cancer and their molecular characterization and clinical significance.

Molecular characterization of CTCs in prostate cancer as surrogate markers for treatment response

Since persistence of ligand-mediated androgen receptor (AR) signaling has been documented in CRPC, abiraterone acetate (AA), an androgen biosynthesis inhibitor, is used to prolong life in patients with CRPC already treated with chemotherapy. Miyamoto and colleagues have shown that measuring AR signaling within CTCs may help guide therapy in metastatic prostate cancer, highlighting the use of CTCs as a liquid biopsy[71]. Leversha and colleagues have shown that FISH analysis of CTCs can be a valuable, non-invasive surrogate for routine tumor profiling in patients with progressive castration-resistant metastatic prostate cancer[72]. Recent results by Darshan and colleagues suggest that monitoring AR subcellular localization in the CTCs of CRPC patients might predict clinical responses to taxane chemotherapy[73]. Moreover, coding mutations in the *AR* gene that represent a possible mechanism underlying the development of CRPC have been identified in tissue samples from patients with advanced prostate cancer, as well as in CTC-enriched peripheral blood samples from CRPC patients[74].

Danila and colleagues studied the role of transmembrane protease, serine 2 (TMPRSS2)-v-ets erythroblastosis virus E26 oncogene homolog (ERG) fusion, an androgen-dependent growth factor, in CTCs as a biomarker of sensitivity to AA[75]. Hormone-driven expression of the ERG oncogene after fusion with TMPRSS2 occurs in 30–70% of therapy-naïve prostate cancers. Molecular profiles of CTCs with an analytically valid assay identified the presence of the prostate

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cancer-specific TMPRSS2-ERG fusion but did not predict for response to AA treatment[75]. Attard and colleagues used multicolor FISH to show that CRPC CTCs, metastases, and prostate tissue invariably had the same ERG gene status as therapy-naïve tumors. They reported a significant association among ERG rearrangements in therapy-naïve tumors, CRPCs and CTCs, and magnitude of PSA decline (p=0.007) in CRPC patients treated with abiraterone acetate[76]. These findings demonstrate the role of CTCs as surrogate markers that can be obtained in a routine practice setting[75].

BRCA1 allelic imbalances were also detected among CTCs in multifocal prostate cancer using FISH analysis[77]. BRCA1 losses in subpopulations of prostate cancer cells might be one confounding factor initiating tumor dissemination and might provide an early indicator of shortened DFS[77]. The utility of CTC enumeration in hormone-sensitive prostate cancer was recently shown by Goodman and colleagues. They enumerated CTCs in 33 consecutive patients undergoing androgen deprivation therapy and reported that initial CTC values predict the duration and magnitude of response to hormonal therapy. CTC enumeration may identify patients at risk of progression to CRPC before initiation of androgen deprivation therapy[78].

Circulating endothelial cells, CTCs, and tissue factor levels alone and combined can predict OS early on in CRPC patients treated with docetaxel-based therapy[79]. Evaluation of the association between circulating objects positive for epithelial cell adhesion molecules and cytokeratin (EpCAM+CK+), that are not counted as CTCs, and survival in patients with prostate cancer has shown that each EpCAM+CK+CD45- class showed a strong association with OS ($p < 0.001$)[80].

Lung cancer

CTC detection in lung cancer in particular has proven difficult to perform, as CTCs in this type of cancer often present with non-epithelial characteristics. Moreover, as many detection methods rely on the use of epithelial markers to identify CTCs, the loss of these markers during EMT in certain metastatic cancers can render these methods ineffective.

Zhu et al. evaluated the presence of *EpCAM/MUC1* mRNA-positive CTCs in 74 non-small cell lung cancer (NSCLC) patients. They showed that disease-free survival and overall survival were significantly reduced in patients with *EpCAM/MUC1* mRNA-positive CTCs, preoperatively and postoperatively[81]. In a recent study, isolation by size of epithelial tumor cells (ISET) and immunofluorescence were used to enrich CTCs by blood filtration in an EpCAM-independent manner. It was shown that hybrid CTCs with an epithelial/mesenchymal phenotype exist in patients with NSCLC and it is believed that their characterization should provide further insight on the significance of EMT in CTCs and on the mechanism of metastasis in patients with NSCLC[82].

Molecular characterization of CTCs in lung cancer as surrogate markers for treatment response

Haber's group showed for the first time that lung cancer patients whose CTCs carried an EGFR mutation known to cause drug resistance had faster disease progression than CTCs lacking the mutation[83]. In late-stage lung cancer patients, *EGFR* mutations were evaluated in single-tumor cells enriched from blood using laser microdissection. Using this technique, individual CTCs were isolated and whole-genome amplification of the DNA was performed by PCR sequencing for the detection of exon 19 microdeletion and L858R and T790M mutations. The amplification success rates were 55% (11/20) for exon 19 deletion, 45% (9/20) for T790M mutation, and 85% (17/20) for L858R mutation. Mutational analysis using a six-gene mutation panel (*EGFR, KRAS, BRAF, NRAS, AKT1*-, and *PIK3CA*) was performed in patients with advanced NSCLC. Only one *EGFR* mutation (exon 19 deletion) was detected in CTC-derived DNA from the 38 patient samples analyzed[84].

The diagnostic test for *ALK* rearrangement in NSCLC for crizotinib treatment is currently performed on tumor biopsies or fine-needle aspirations. Pailler et al. recently evaluated whether or not *ALK* rearrangement diagnosis could be performed using CTCs. They found that *ALK* rearrangement can be detected in CTCs of patients with *ALK*-positive NSCLC by using a filtration technique and FISH, enabling both diagnostic testing and monitoring of crizotinib treatment. These results clearly suggest that CTCs harboring a unique *ALK* rearrangement and mesenchymal phenotype may arise from clonal selection of tumor cells that have acquired the potential to drive metastatic progression of *ALK*-positive NSCLC[85]. Table 5 lists CTCs in lung cancer and their molecular characterization and clinical significance.

Colorectal cancer

The prognostic value of CTCs and DTCs (disseminated tumor cells) in patients with resectable colorectal liver metastases or widespread metastatic colorectal cancer (mCRC) has been clearly shown in a meta-analysis study that was based on 12 studies[86].

Table 5. Molecular characterization and clinical significance of CTCs in lung cancer.

Type of cancer	Detection method used	Biomarker used	Prognostic significance	References
Early NSCLC	FISH	*ALK* rearrangement, cytokeratins, E-cadherin, vimentin, N-cadherin	Not evaluated	85
	RT-PCR	*EpCAM/MUC1* mRNA	OS: yes DFS: yes	81
Advanced NSCLC	ISET, IF	CK, vimentin	OS: not evaluated PFS: not evaluated	82
	Allele-specific PCR	*EGFR* mutations	OS: not evaluated DFS: yes	83
	CellSearch, RT-PCR, ARMS real-time PCR	Enumeration of CTCs, *EGFR* expression, *EGFR* mutations	OS: N.V. PFS: yes	84

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Table 6. Molecular characterization and clinical significance of CTCs in colorectal cancer.

Type of cancer	Detection method used	Biomarker used	Prognostic significance	References
Non-metastatic colorectal cancer	CellSearch	Enumeration of CTCs	Not evaluated	94
	RT-qPCR	*CEA/CK/CD133*	OS: yes DFS: yes	101
Metastatic colorectal cancer	CellSearch	Enumeration of CTCs	OS: yes PFS: yes	87–89
	RT-qPCR	*CK-19, MUC1, EPCAM, CEACAM5* and *BIRC5*	OS: not evaluated PFS: yes	90
	Sequencing	*KRAS/PIK3CA* mutations	Not evaluated	96
	CTC isolation, DEPArray	*KRAS* mutations	Not evaluated	97
	Allele-specific blocker PCR	*KRAS* and *BRAF*	Not evaluated	95
	Fluorescent immunocytochemistry	PLS3	OS: yes PFS: yes	100
	Next-generation sequencing	*APC, KRAS*, and *PIK3CA* mutations	Not evaluated	98

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Metastatic colorectal cancer

In a prospective multicenter study, CTCs were enumerated using the CellSearch system in 430 patients with mCRC at baseline, and after starting first-, second-, or third-line therapy. According to this study, the number of CTCs before and during treatment was an independent predictor of PFS and OS in patients with mCRC. Based on these results, the CellSearch assay was cleared by the FDA for mCRC[87]. It was further shown that CTC enumeration before and during treatment independently predicted PFS and OS in advanced colorectal cancer patients treated with chemotherapy plus targeted agents, and provides information in addition to CT imaging[88]. The clinical utility of CTC enumeration in improving the clinician's ability to accurately assess oxaliplatin-based chemotherapy treatment benefit, and in expediting the identification of effective treatment regimens for individual patients was further shown[89]. Another study showed a strong correlation between CTC detection and radiographic disease progression in patients receiving chemotherapy for colorectal cancer[90]. The impact of immediate and differing surgical interventions on CTCs and their compartmentalization or localization in different anatomic vascular sites was evaluated. Surgical resection of metastases, but not radiofrequency ablation, was shown to immediately decrease CTC levels[91]. Another recent study showed that the qualitative and quantitative detection of CTCs is higher in the mesenteric venous blood compartments of patients with mCRC[92].

Non-metastatic colorectal cancer

The prognostic role of CTCs in non-metastatic colorectal cancer is less clear than in mCRC. The low abundance of CTCs in non-metastatic colorectal cancer requires very sensitive and specific detection methods. A recent review examined the possible clinical significance of CTCs in non-metastatic colorectal cancer (TNM-stages I–III) with the primary focus on detection methods and prognosis. According to the findings reported, the presence of CTCs in peripheral blood is a potential marker of poor disease-free survival in patients with non-metastatic colorectal cancer[93]. CTC detection might help in the selection of high-risk stage II colorectal cancer patient candidates for adjuvant chemotherapy, after enumerating CTCs with the FDA-cleared CellSearch system[94].

Molecular characterization of CTCs in colorectal cancer as surrogate markers for treatment response

Molecular characterization of CTCs could provide important information for improving the management of colorectal cancer patients. Table 6 lists CTCs in colorectal cancer and their molecular characterization and clinical significance.

The presence of *KRAS* and *BRAF* mutations is currently assessed in the primary tumor, since it has been shown to reflect the efficacy of anti-EGFR therapy in metastatic colorectal cancer. It was interesting to find discordance among primary tumors, CTCs, and metastatic tumors with respect to the mutation status of *KRAS* and *BRAF* in metastatic colorectal cancer patients[95]. Using the CellSearch system, *EGFR* expression, *EGFR* gene amplification, and *KRAS, BRAF*, and *PIK3CA* mutations were shown in single CTCs of patients with metastatic colorectal cancer[96]. When *KRAS* mutations were detected in metastatic colorectal cancer after isolating single CTCs with the DEPArray, there was a mutational concordance between CTCs and primary tumors in 50% of matched cases[97]. Using deep sequencing, mutations in *APC, KRAS*, and *PIK3CA* genes that were found in CTCs were also present at subclonal levels in the primary tumors and metastases from the same patient[98]. *KRAS* mutation status was also examined in CTCs of metastatic colorectal cancer patients[99].

In a very recent and interesting study, it was shown that Plastin3 is a novel marker for CTCs undergoing EMT and is associated with colorectal cancer prognosis, particularly in patients with Dukes' B and Dukes' C[100]. Patients with CTC positivity at baseline had a significantly shorter median PFS compared with patients with no CTCs. A significant correlation was also found between CTC detection during treatment and radiographic findings at the six-month staging[90]. Iinuma et al. studied the mRNA expression of *CEA, CK-19, CK-20*, and/or *CD133* in CTCs from 735 patients with CRC. They reported that the OS and DFS of patients with Dukes' stage B or C cancer, who were positive for *CEA/CK/CD133*, were significantly worse than the OS and DFS of patients who were negative for these markers[101].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a primary liver cancer that is very challenging in terms of its complex etiology and management. Currently, some interesting and encouraging

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results have been achieved in HCC CTC detection, although the knowledge about the clinical relevance of CTCs in HCC is lagging behind other major tumor types. Zhang et al. have recently reviewed existing and developing methodologies for CTC detection, and describe the potential clinical impact of the identification and molecular characterization of CTCs in HCC patients[102]. Very recently, Nel et al. assessed which non-hematopoietic cell types were identifiable in the peripheral blood of HCC patients, and whether their distribution during treatment courses is associated with clinical characteristics. Their data have shown a remarkable variation of cells with epithelial, mesenchymal, liver-specific and mixed characteristics, as well as different size ranges. The distribution of these subgroups varied significantly between different patient groups and was associated with therapeutic outcome[103]. Schulze et al. investigated the prognostic relevance of EpCAM-positive CTCs in 59 patients with HCC with the CellSearch system. Their study demonstrated frequent presence of EpCAM-positive CTCs in patients with intermediate or advanced HCC and its prognostic value for OS, with possible implications for future treatment stratification[104]. In HCC patients undergoing curative resection, stem cell-like phenotypes were observed in EpCAM+ CTCs. As well, a preoperative CTC number of $\geq$2 cells/7.5 mL was found to predict for tumor recurrence in HCC patients after surgery, especially in patient subgroups with AFP levels of $\leq$400 ng/mL or low tumor recurrence risk[105].

Pancreatic cancer

The poor prognosis of pancreatic cancer patients is associated with the frequent and early dissemination of the disease, as well as late detection due to unspecific and late symptoms from the primary tumor. Pancreatic cancers frequently spread to the liver, lung, and skeletal system, suggesting that pancreatic tumor cells must be able to intravasate and travel through the circulation to distant organs. Detection of CTCs in peripheral blood may be a promising biomarker for the detection and prognosis of pancreatic cancer. In a very recent review on the clinical relevance of CTC detection in pancreatic cancer, Tjensvoll et al. reported evidence that the presence of CTCs correlates with an unfavorable outcome[106]. Bidard et al. reported that CTC detection appears as a promising prognostic tool in locally advanced pancreatic carcinoma (LAPC) patients. In this study, CTC detection rates and prognostic value were evaluated in a prospective cohort of LAPC patients using the CellSearch system. CTC positivity was associated with poor tumor differentiation and with shorter OS in multivariable analysis[107]. A very recent meta-analysis aimed to assess the prognostic value of CTCs in patients with pancreatic cancer, including nine cohort studies with a total of 623 pancreatic cancer patients, 268 CTC-positive, and 355 CTC-negative. This meta-analysis revealed that patients in the CTC-positive group were significantly associated with poor PFS. Furthermore, pancreatic cancer patients in the CTC-positive group also showed worse OS than those in the CTC-negative group[108]. Larger studies, as well as characterization of the CTC population, are required to achieve further insight into the clinical implications of CTC detection in pancreatic cancer patients.

Head and neck cancer

According to a prospective clinical follow-up study of patients with squamous cell carcinoma of the head and neck (SCCHN) undergoing surgical intervention, patients with no detectable CTCs had a significantly higher probability of DFS[109]. The same group has shown recently that in patients with SCCHN, the presence of CTCs correlates with worse disease-free survival[110]. This conclusion was based on the results obtained after isolation of CTCs by a purely negative enrichment methodology which does not depend on the expression of surface epithelial markers. According to another prospective multicentric analysis that studied the possible role of CTC identification in locally advanced head and neck cancer (LAHNC), CTCs were frequently identified in oro- and hypopharyngeal cancer and in sinonasal undifferentiated carcinoma (SNUC). A decrease in the number of CTCs or their absence throughout the treatment also seemed to be related to non-progressive disease, after either complete or incomplete remission, and with the proportion of patients alive and no evidence of disease[111].

Bladder cancer

The finding of extravesical and node-positive disease at the time of radical cystectomy for patients with clinically localized bladder cancer is not uncommon, and is due to imprecise clinical staging. CTCs have been shown to be present in the peripheral blood of patients with metastatic urothelial carcinoma. Guzzo et al. evaluated the ability of CTCs to predict extravesical disease in bladder cancer patients prior to radical cystectomy. They came to the conclusion that CTC status is not likely to be a clinically useful parameter for directing therapeutic decisions in patients with $\leq$cT2 bladder cancer[112]. Rink et al. prospectively detected and evaluated the biological significance of CTCs in patients with bladder cancer, especially in those patients with non-metastatic, advanced bladder cancer using the CellSearch system. Their findings suggest that the presence of CTCs may be predictive for early systemic disease since CTCs were detected in 30% of patients with non-metastatic disease[113]. Gradilone et al. have chosen to evaluate the prognostic significance of survivin-expressing CTCs in patients with T1G3 bladder tumors since the prognosis of T1G3 bladder cancer is highly variable and unpredictable from clinical and pathological prognostic factors. They reported that the presence of CTCs was an independent prognostic factor for DFS in patients with T1G3 bladder cancer[114].

Melanoma

Blood-based assays to detect melanoma progression by monitoring levels of CTCs and cfDNA can be used to evaluate progress and therapy response in melanoma patients[115]. Advances in the molecular analysis of CTCs may provide insight into new avenues of approaching therapeutic options that would benefit personalized melanoma management[116]. Mutated *BRAF* was detected in 81% of 21 assessed stage IV melanoma patients[117]. Single, isolated CTCs from patients with melanoma have been subjected to

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BRAF and *KIT* mutational analysis. The *BRAF* sequences and *KIT* sequences identified in CTCs were inconsistent with those identified in autologous melanoma tumors, showing clonal heterogeneity[118]. The expression of *MART-1, MAGE-A3*, and *PAX3* mRNA biomarkers has been evaluated by RT-qPCR in stage IV melanoma patients. CTC biomarker(s) ($\geq$1) were detected in 54% of patients and were significantly associated with disease-free survival and overall survival[119].

Conclusions and future challenges

The main advantage of CTC analysis is based on the unique potential of CTCs to offer a minimally invasive ''liquid biopsy'' sample, easily obtainable at multiple time points during disease progression. This can provide valuable information on the very early assessment of treatment efficacy and can help towards establishing individualized treatment approaches that will improve efficacy with less cost and side effects for cancer patients.

Further research on the molecular characterization of CTCs will provide important information for the identification of therapeutic targets and understanding resistance to therapies. The molecular characterization of CTCs is highly promising especially in combination with next-generation sequencing technologies that will enable the elucidation of molecular pathways in CTCs, and will probably lead to the design of novel molecular therapies specifically targeting CTCs[98]. Even if this is still far from being considered for application in a routine clinical setting, it holds great promise for the future management of cancer patients.

However, even if the clinical significance of CTC detection has been revealed in many clinical studies so far, there is still a lot to be done for the automation, standardization, quality control, and accreditation of analytical methodologies used for the isolation, detection, and molecular characterization of CTCs, before their implementation in routine clinical practice[7].

In conclusion, the clinical use of CTCs as a ''liquid biopsy'', for selection of patients and real-time monitoring of therapies, will have a major impact on personalized medicine in many types of solid cancers.

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