The Role of CTCs as Tumor Biomarkers

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Abstract

Detection of Circulating Tumor Cells (CTCs) in peripheral blood can serve as a "liquid biopsy" approach and as a source of valuable tumor markers. CTCs are rare, and thus their detection, enumeration and molecular characterization are very challenging. CTCs have the unique characteristic to be non-invasively isolated from blood and used to follow patients over time, since these cells can provide significant information for better understanding tumour biology and tumour cell dissemination. CTCs molecular characterization offers the unique potential to understand better the biology of metastasis and resistance to established therapies and their analysis presents nowadays a promising field for both advanced and early stage patients. In this chapter we focus on the latest findings concerning the clinical relevance of CTC detection and enumeration, and discuss their potential as tumor biomarkers in various types of solid cancers. We also highlight the importance of performing comparison studies between these different methodologies and external quality control systems for establishing CTCs as tumor biomarkers in the routine clinical setting.

Keywords

Breast cancer • Cancer stem cells • Circulating tumor cells (CTC) • Circulating tumour stem cells • CK-19 • Colorectal cancer • Epcam • Gastrointestinal Cancers • Hepatocellular carcinoma • Individualized treatment • Liquid biopsy • Lung cancer • Melanoma • Migrating cancer stem cells • Molecular characterization • Non-small-cell lung cancer (NSCLC) • Oncoproteomics • Overall survival (OS) • Pancreatic cancer • Peripheral blood • Predictive biomarkers • Prognostic biomarkers • Progression free survival (PFS) • Prostate cancer • Tumor biomarkers • Solid cancer

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Abbreviation

AA	Abiraterone acetate
AR	Androgen receptor
BC	Breast cancer
CRPC	Castration-resistant prostate cancer
CA-15-3	Cancer antigen 15–3
CEA	Carcinoembryonic antigen
cfDNA	Cell free DNA
CTCs	Circulating Tumor Cells
CK-19	Cytokeratin-19
CK-7	Cytokeratin-7
DFS	Disease Free Survival
DTC	Disseminated tumor cells
EGFR	Epidermal growth factor receptor
EMT	Epithelial-Mesenchymal Transition
EQA	External quality assurance
CNA	Genome-wide copy-number aberration
HCC	Hepatocellular carcinoma
hTERT	Human telomerase reverse transcriptase
ISET	Isolation by size of epithelial tumour cells
LAPC	Locally advanced pancreatic carcinoma
LAHNC	Locally advanced head and neck cancer
LOH	Loss of heterozygosity
MBC	Metastatic breast cancer
mCRC	Metastatic colorectal cancer
NIH	National Institutes of Health
NSCLC	Non small cell lung cancer
OS	Overall Survival
PE	Pleural Effusion
PFS	Progression Free Survival
PSA	Prostate Specific Antigen
RT-PCR	Reverse transcriptase-polymerase chain reaction
SLN	Sentinel lymph node
SNP	Single-nucleotide polymorphism
SNUC	Sinonasal undifferentiated carcinoma
SCCHN	Squamous cell carcinoma of head and neck
TTF-1	Thyroid transcription factor 1
TGF-P	Transforming growth factor
TMPRSS2	Transmembrane protease serine 2

The major cause of cancer mortality is tumor metastasis and therefore there is a compelling need for the discovery and validation of novel biomarkers for cancer screening, diagnosis, prognosis and therapeutic monitoring [29]. The development of noninvasive methods to detect and monitor tumors continues to be a major challenge in oncology and the search for new and better non-invasive tumor biomarkers has become a holy grail of contemporary cancer research. As Dr Diamandis correctly has pointed out, "the journey of a cancer biomarker from the bench to the clinic is long, difficult and challenging and every step needs to be very carefully planned and executed in detail to succeed" [110].

The presence of tumor cells, circulating in blood of cancer patients was first reported by Thomas Ashworth in 1869 [5]. Nowadays, almost 150 years after this first report, the clinical and research potential of Circulating Tumor Cells (CTCs) is becoming widely recognized [118]. CTCs are indicators of residual disease and thus pose an increased risk of metastasis and poorer outcomes to those patients who are CTC-positive. The number of studies on CTCs published in peer reviewed journals is constantly rising during the last 15 years (Fig. 21.1).

CTCs represent cells that are shed in the circulation by primary or metastatic tumors and thus provide a "liquid biopsy" approach that enables frequent samplings of a patient's tumor and follow-up of patients during treatment. CTCs are in principle very different from all other established tumor biomarkers, since they represent a unique source of valuable information. By studying CTCs we can better understand tumour biology and tumour cell dissemination while their molecular characterization offers the unique potential to understand resistance to established therapies [79, 81].

CTC analysis is extremely challenging since CTCs are very rare, and the amount of available sample is very limited. Since CTC detection was shown to be of considerable utility in the clinical management of patients with solid cancers, a big variety of analytical systems for their isolation and detection have been developed [80, 108, 109, 163]. New areas of research are directed towards

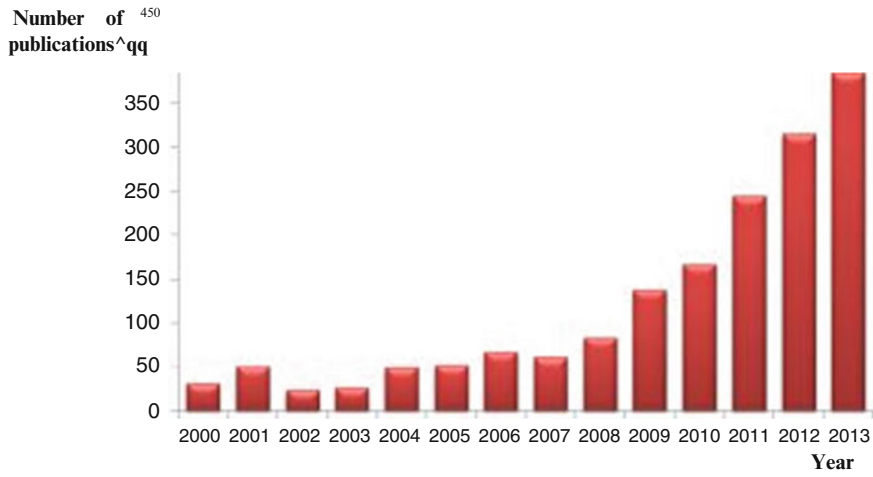

Fig. 21.1 Publications on CTCs during the last 13 years (http://www.ncbi.nlm.nih.gov/pubmed)

developing novel assays for CTC molecular characterization [3, 80, 120]. A high heterogeneity of CTC even among the same individuals has been observed by performing high dimensional single CTC profiling, and directly measuring gene expression in individual CTC without the common practice of pooling such cells [120]. Molecular studies on CTCs have often been limited by a low number of CTCs isolated from a high background of leukocytes. Improved enrichment techniques are now allowing molecular characterization of single CTCs, whereby molecular markers on single CTCs may provide a real-time assessment of tumor biomarker status from a blood test or "liquid biopsy", potentially eliminating the need for a more invasive tissue biopsy.

However, many questions still remain unanswered regarding the biology of CTC, the optimal method to enumerate and characterize them and the path to regulatory and general clinical acceptance of technology platforms currently under development [109].

In this chapter we focus on the latest findings concerning the clinical relevance of CTC detection and enumeration, and discuss their potential as tumor biomarkers in various types of solid cancers. We also discuss the different platforms available for CTC isolation, enumeration and molecular characterization, and highlight the importance of performing comparison studies between these different methodologies. Finally we discuss the importance of external quality control systems for establishing CTCs as tumor biomarkers in the routine clinical setting.

21.1 CTCs as Tumor Biomarkers

The clinical significance of CTC has been evaluated in many types of solid cancers, and the CTC enumeration test in metastatic breast, colorectal and prostate cancer has been cleared by the FDA almost a decade ago. There is a significant interest nowadays on examining CTCs as "surrogate" markers for potentially improved survival for regulatory purposes, and as prognostic or predictive biomarkers in a variety of solid cancers. In the official website of the National Institutes of Health (http://clinicaltrials.gov/ct2/home) our search (May 2014) based on the key word "Circulating Tumor Cells", revealed 587 ongoing clinical studies; when we searched for specific cancer types, a whole spectrum of studies evaluating the role of CTC as surrogate biomarkers was revealed (Fig. 21.2). These trials have different designs in various patient populations but are expected to be the pivotal trials for CTC implementation in the routine management of cancer patients [12, 13, 74]. The American Society of Clinical Oncology (ASCO) cited CTC and DTC

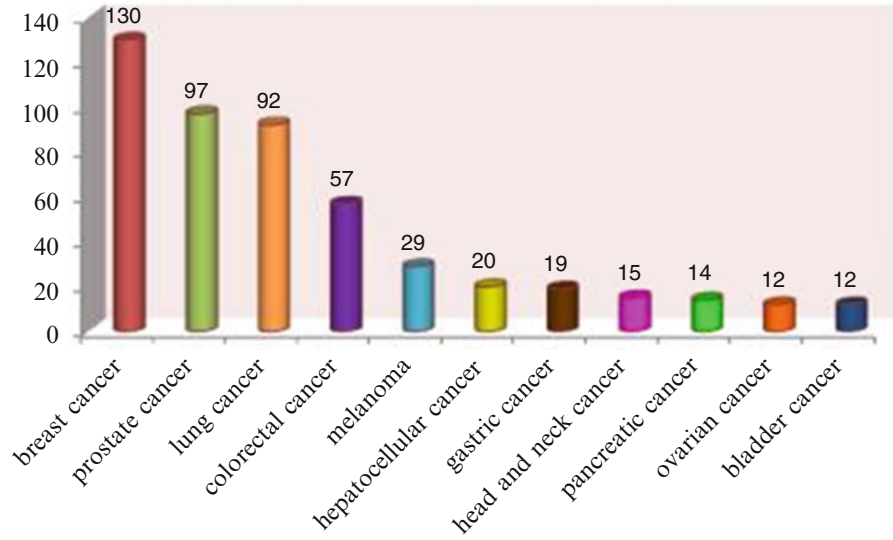

Fig. 21.2 Clinical studies that include CTCs analysis: in the official website of the National Institutes of Health (http://clinicaltrials.gov/ct2/home) our search on May 2014 on clinical studies, based on the key word "Circulating Tumor Cells", revealed 587 ongoing clinical studies

for the first time in its 2007 recommendations on tumor markers, however in the category of insufficient evidence to support routine use in clinical practice [51]. However, very recently, the American Joint Committee on Cancer has proposed a new category, M0(i+), for TNM staging in breast cancer (BC) defined as "no clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells (no larger than 0.2 mm) in blood, bone marrow, or other non-regional nodal tissue in a patient without symptoms or signs of metastases".

Below we are presenting the main studies performed so far on the clinical evaluation of CTCs as tumor biomarkers in various types of solid cancers.

21.1.1 Breast Cancer

In the official website of the NIH our search (May 2014) based on the key word "Circulating Tumor Cells AND breast cancer" revealed 130 studies (Fig. 21.2). The first comprehensive meta-analysis of published literature on the prognostic relevance of CTC in patients with early-stage and metastatic breast cancer clearly indicated that the detection of CTC is a reliable prognostic factor [164, 165].

21.1.1.1 Metastatic Breast Cancer

Cristofanilli and colleagues have shown by using the CellSearch System (Veridex, USA) that CTC 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 CTC/7.5 ml of blood in these patients was highly predictive of clinical outcome [22]. This paper revolutionized the clinical applications of CTC in many types of cancer, since it led to the FDA clearance of the CellSearch assay that is standardized, semi-automated and not subjected to preanalytical errors. Since then, a plethora of clinical studies has verified the importance of CTC enumeration in metastatic breast cancer [41, 42, 98, 117, 155]. Liu et al. conducted a prospective study to demonstrate that CTC results correlate strongly with radiographic disease progression at the time of and in advance of imaging. They provided the first evidence of a strong correlation between CTC results and radiographic disease progression

in patients receiving chemotherapy or endocrine therapy for MBC. These findings support the role of CTC enumeration as an adjunct to standard methods of monitoring disease status in MBC [85].

A very recent study assessed the clinical validity of CTC quantification for prognostication of patients with metastatic breast cancer by undertaking a pooled analysis of 1944 individual patient data. The authors contacted 51 European centers and asked them to provide reported and unreported anonymized data for individual patients with metastatic breast cancer starting a new line of therapy concerning PFS or OS, or both, and CTC quantification by the Cell Search method at baseline (before start of new treatment). The authors report that increased CTC counts 3–5 weeks after start of treatment, adjusted for CTC count at baseline, were associated with shortened PFS. Survival prediction was significantly improved by addition of baseline CTC count to the clinicopathological models The data collected confirmed the independent prognostic effect of CTC count on PFS and OS. CTC count also improves the prognostication of MBC when added to full clinic0pathological predictive models, whereas serum tumor markers do not. *CEA* and *CA 15–3* concentrations at baseline and during therapy did not add significant information to the best baseline model. [14].

In another recent prospective multicenter study a total of 254 MBC patients were enrolled at first diagnosis of metastatic disease or disease progression (before the start of a new treatment regimen). By using an EpCAM-independent enrichment approach, viable CTC releasing CK-19 as an epithelial cell marker were detected in the peripheral blood by the EPISPOT assay, and the FDA cleared CellSearch was used as the reference method. CTC detection using the EPISPOT assay has shown prognostic relevance of the presence of viable CTC. Interestingly, the combination of the EPISPOT and CellSearch assays was the strongest predictor of OS [127].

The presence of CTCs was found to be an effective measure of treatment efficacy and immune system function in MBC patients [18]. Green et al. report that that those patients with greater than 5 CTCs per 7.5 mL blood had significantly decreased responses by their immune cells when compared with those patients who had 5 CTCs or less. They also verified the already reported by many other groups correlation between disease progression and CTC-positive patients, indicating that those who have a positive test should be closely monitored by their clinician [46].

The detection and prognostic significance of CTCs in MBC in respect to the different immunehistochemical subtypes of breast cancer has been also recently evaluated. Peeters et al. report that the detection of EpCAM positive CTCs was not clearly associated with any of the immunehistochemical subtypes of breast cancer in patients with MBC before first-line treatment. Their data also suggest a lower prognostic significance of CTC evaluation in *HER2*-positive patients with MBC [112]. The French group, led by JY Pierga specifically evaluated the impact of CTC on brain metastasis outcome and has shown that there is a correlation between CNS metastasis response, outcome and early CTC clearance under targeted treatment of *HER2* positive MBC [115].

21.1.1.2 Early Breast Cancer

The prognostic value of CTC in axillary lymph node-negative breast cancer patients, based on a nested RT-PCR was already shown in 2002 [142]. By using a real time RT-qPCR assay for CK-19 mRNA [140, 141] CTC detection was shown to be an independent prognostic factor for reduced DFI and OS before [158], during [157] and after [156] chemotherapy in early breast cancer. Detection of CTC before adjuvant chemotherapy predicted for poor clinical outcome mainly in patients with ER-negative, triple-negative, and HER2-positive early-stage breast cancer [59]. When CTC were prospectively detected before and after neoadjuvant chemotherapy in a phase II trial it was found that detection of one or more CTC in 7.5 ml of blood before neoadjuvant chemotherapy can accurately predict OS [116]. A more recent study investigating the value of CTC detection during the first 5 years of follow-up in predicting late disease relapse, has shown that persistent detection of CTC was associated with an increased risk of late disease relapse and death

in patients with operable breast cancer and indicates the presence of chemo-and hormonotherapy-resistant residual disease [133]. Lucci et al., prospectively collected data on CTC at the time of definitive surgery from chemonai've patients with stage 1–3 breast cancer. They enumerated CTC and assessed outcomes at a median follow-up of 35 months, and have shown that the presence of one or more CTC predicted for early recurrence and decreased overall survival in chemonai've patients with non-metastatic breast cancer [88].

These results were also recently confirmed by another study that was based on an RT-PCR molecular assay for CTC detection, the AdnaTest BreastCancer™ (AdnaGen AG, Germany). This assay is based on the detection of *EpCAM, HER2* and *MUC1* specific transcripts in enriched CTC-lysates. Mikulova et al. report that CTCs were detected in the peripheral blood of approximately 31 % of early stage breast cancer patients before therapy, while only 7 % of all patients remained CTCs positive after adjuvant therapy. There was no correlation between CTCs and tumor size, tumor grade, histological grade and receptor status [94].

21.1.1.3 CTC as Surrogate Markers for Treatment Response in Breast Cancer

Based on the current guidelines, in breast cancer, hormone therapy and anti-HER-2 therapies are prescribed according to the hormone (ER/PR expression) and *HER-2* status of the primary tumor. However, a growing body of evidence is showing that the hormone receptor and *HER-2* status in CTC can be different from that in the primary tumors and even change over time, especially during disease recurrence or progression in breast cancer patients [33, 34, 58, 122, 129, 139]. Based on that, re-evaluation of hormone receptor and HER-2 status by molecular characterization of CTC is a strategy with potential clinical application. An optimal individualized treatment could be selected by characterizing *ER* and *HER-2* status in CTC and comparing it to the primary tumor [124, 154].

Many research groups have already shown that HER2-positive CTCs can be detected in patients with HER2-negative primary tumors [34, 35, 58, 114, 122]. Ligthart et al. have recently developed an automated algorithm for evaluating HER-2 expression in CTC when using the CellSearch system. They report that HER-2 expression is very heterogeneous among CTC within each patient [82]. Georgoulias et.al were the first to investigate the effect of trastuzumab in HER2-negative patients that have CK(+)/HER2-positive CTC in a randomized phase II study. According to their results, administration of trastuzumab can eliminate chemotherapy-resistant CK19 mRNA-positive CTCs, reduce the risk of disease recurrence and prolong Disease Free Survival (DFS) [39].

However, to evaluate CTCs as a predictive biomarker and obtain clinically meaningful results large studies that are specifically designed around effective therapies are needed. This is very challenging, and difficult, because of the high cost and continuous changes in the molecular targeted therapies. Very recently the TREAT-CTC study (http://clinicaltrials.gov/ct2/results?term=TREAT-TC+study&Search=Search), is a randomized phase II trial for patients with HER2 negative primary BC who after completing (neo) adjuvant chemotherapy and surgery have detectable CTC in peripheral blood. The aim of the study is to see whether *HER2* directed therapy reduces relapses in women at high risk of recurrence, and for this reason women positive for CTC detection, as evaluated by using the CellSearch system, after neoadjuvant chemotherapy are randomly assigned to trastuzumab or a placebo. Moreover, the fact that breast cancer is a disease with clearly dinstict molecular subtypes[113] could be a reason why specific CTC counts or molecular phenotypes that are predictive for response to one therapy are not relevant for others.

Epithelial-Mesenchymal Transition (EMT) is an essential process in the metastatic cascade [10, 83]. CTC molecular characterization is highlighting the importance of EMT, a process which may be crucial for allowing tumors to invade into and grow at sites distant from the original site of tumor. The expression levels of EMT-inducing transcription factors have been determined in CTC in primary breast cancer patients [93].

Investigation of the apoptotic and proliferative status in CTC of breast cancer patients has shown that patients with metastatic and advanced disease had significantly lower numbers of apoptotic CTCs compared to patients with early breast cancer and that adjuvant chemotherapy reduced both the number of CTCs per patient and the number of proliferating CTCs [66]. Very recently Yu et al. have shown by serial monitoring of CTC in patients with breast cancer that these cells simultaneously expressed mesenchymal and epithelial markers, and that mesenchymal cells expressing known EMT regulators, including transforming growth factor (TGF)-P pathway components and the *FOXC1* transcription factor were associated with disease progression [162].

Similarly, the detection of CTCs expressing markers of stemness may also have important implications for treatment resistance. A major proportion of CTC of metastatic breast cancer patients show EMT and tumor stem cell characteristics [2] and CTC expressing *TWIST* and vimentin, were identified in patients with metastatic and early breast cancer patients [67]. 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 [149]. Currently used detection methods for CTC are not efficient to identify this subtype of CTC which underwent EMT [68].

Moreover studies on the molecular characterization of CTCs have revealed that CTCs even within the same patient are heterogeneous. In non-metastatic breast cancer patients the expression of estrogen, progesterone and epidermal growth factor receptor (*EGFR*) by immunofluorescence experiments revealed heterogeneous expression of these hormonal receptors in samples from the same patients [100].

21.1.2 Prostate Cancer

In prostate cancer, CTC enumeration has been extensively studied and validated as a prognostic tool and has received FDA clearance for use in monitoring advanced disease. In the official website of the National Institutes of Health (http://clinicaltrials.gov/ct2/home) our search on May 2014 on clinical studies, based on the key word "Circulating Tumor Cells AND prostate cancer" revealed 97 studies. In patients with advanced prostate cancer, CTC enumeration by using the Veridex CellSearch™ system, at baseline and post-treatment, has been cleared by the FDA for quantifying the load of tumour cell dissemination. This test is prognostic of survival and is currently being implemented into routine clinical practice for estimating prognosis and monitoring treatment success [136]. 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 CTC as potential predictive biomarkers of tumor sensitivity to a therapeutic modality [23, 24].

The main CTC studies in advanced and localized prostate cancer, highlighting the important gains as well as the challenges posed by various approaches, and their implications for advancing prostate cancer management have been recently reviewed in detail [57].

21.1.2.1 Metastatic Prostate Cancer

Moreno et al. were the first to report 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 CTC correlates with disease progression with no diurnal variations [96]. Later, in 2007, Danila and colleagues reported that the number of CTC before therapy provides unique information relative to prognosis and that the shedding of cells into the circulation represents an intrinsic property of the tumor, distinct from the extent of the disease [25]. In 2008, data presented by de Bono and colleagues showed that CTC enumeration by using the CellSearch™ system has prognostic and predictive value in patients with metastatic castration- resistant prostate cancer (CRPC) and is an independent predictor of OS in CRPC, opening the way to the FDA clearance of this assay for the evaluation of CRPC [28]. CTC numbers, analyzed as a continuous variable, predict OS and provide independent prognostic information to time to disease

progression and can be used to monitor disease status [105, 134].

Resel and colleagues analyzed the correlation between CTC levels and the Prostate Specific Antigen (PSA) level, Gleason score, and TNM stage in patients with metastatic hormone-sensitive prostate cancer and reported that 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 [128]. Combination of CTC and *PSA* velocity may offer insights into the prognosis and management of advanced PC [53, 131].

CTC enumeration was very recently prospectively validated in standard first-line docetaxel treatment for metastatic CRPC. S0421, a phase III trial of docetaxel plus prednisone with or without atrasentan, validated the prognostic utility of CTC enumeration for OS and disease response. Baseline CTC counts were prognostic, and rising CTCs at 3 weeks heralded significantly worse OS, potentially serving as an early metric to help redirect and optimize therapy in this clinical setting [43].

21.1.2.2 Early-Stage Prostate Cancer

Recently CTCs have been detected in early prostate cancer and may be a new surrogate candidate towards the decision whether to offer systemic or local treatment [31]. CTC tests may assist with clinical decision-making according to a pilot study that investigated whether CTC could be detected in early-stage prostate cancer patients receiving salvage radiotherapy using the CellSearch system. The results of this study demonstrated that CTC 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 [86]. Recent trials in patients with CRPC are incorporating the detection of CTC, imaging, and patient-reported outcome biomarkers in order to improve future drug development and patient management for patients [135].

21.1.2.3 CTCs as Surrogate Markers for Treatment Response in Prostate Cancer

Prostate cancer growth depends on androgen receptor (AR) signaling. Androgen ablation therapy induces expression of constitutively active androgen receptor splice variants that drive disease progression. Taxanes are a standard of care therapy in CRPC; A very recent study suggests that two clinically relevant AR splice variants, ARv567 and ARv7, differentially associate with microtubules and dynein motor protein, thereby resulting in differential taxane sensitivity in vitro and in vivo. They suggest that androgen receptor variants that accumulate in CRPC cells utilize distinct pathways of nuclear import that affect the antitumor efficacy of taxanes, suggesting a mechanistic rationale to customize treatments for patients with CRPC, which might improve outcomes [148].

Moreover, since persistence of ligand-mediated AR signaling has been documented in CRPC, abiraterone acetate (AA), an androgen biosynthesis inhibitor, was shown to prolong life in patients with CRPC already treated with chemotherapy. Miyamoto and colleagues have shown that measuring AR signaling within CTC may help to guide therapy in metastatic prostate cancer and highlight the use of CTC as liquid biopsy [95]. Leversha and colleagues have shown that FISH analysis of CTC can be a valuable, noninvasive surrogate for routine tumor profiling in patients with progressive castration-resistant metastatic prostate cancer [78]. Recent results by Darshan and colleagues suggest that monitoring AR subcellular localization in the CTC of CRPC patients might predict clinical responses to taxane chemotherapy [26]. 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 and have been also identified in CTC-enriched peripheral blood samples from CRPC patients [64].

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 CTC as a biomarker of sensitivity to AA [23, 24]. Hormone-driven expression of the ERG oncogene after fusion with *TMPRSS2* occurs in 30–70 % of therapy-naive prostate cancers. Molecular profiles of CTC with an analytically valid assay identified the presence of the prostate cancer-specific *TMPRSS2-ERG* fusion but did not predict for response to AA treatment [23, 24]. Attard and colleagues have used multicolor FISH to show that CRPC CTC, metastases, and prostate tissue invariably had the same ERG gene status as therapy-naive tumors and reported a significant association between ERG rearrangements in therapy-naive tumors, CRPC, and CTC and magnitude of PSA decline ($P=0.007$) in CRPC patients treated with abiraterone acetate [6]. These findings demonstrate the role of CTC as surrogate marker that can be obtained in a routine practice setting [23, 24].

BRCA1 allelic imbalances were also detected among CTC in multifocal prostate cancer by using FISH analysis [9]. Especially, *BRCA1* losses might be one confounding factor initiating tumor dissemination and might provide an early indicator of shortened DFS [9]. The utility of CTC enumeration in hormone sensitive prostate cancer was recently shown by Goodman and colleagues, who enumerated CTC 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 [44].

Circulating endothelial cells, CTC and tissue factor levels alone and combined can predict early on OS in CRPC patients treated with docetaxel-based therapy [144]. Evaluation of the association between circulating objects positive for epithelial cell adhesion molecules and cytokeratin (EpCAM+CK+) that are not counted as CTC and survival in patients with prostate cancer has shown that each EpCAM+CK+CD45- circulating object showed a strong association with OS ($P<0.001$) [21].

21.1.3 Lung Cancer

Lung cancer is the leading cause of cancer-related death worldwide. In the official website of the National Institutes of Health (http://clinicaltrials.gov/ct2/home) our search on May 2014 on clinical studies, based on the key word "Circulating Tumor Cells AND lung cancer" revealed 92 studies (Fig. 21.2). 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.

21.1.3.1 Non-Small-Cell Lung Cancer (NSCLC)

Non-small-cell lung cancer (NSCLC) lacks validated biomarkers to predict treatment response. Zhu et.al evaluated the presence of EpCAM/MUC1 mRNA-positive CTCs in 74 non small cell lung cancer (NSCLC) patients and showed that DFS and OS was significantly reduced in patients with EpCAM/MUC1 mRNA-positive CTC preoperatively and postoperatively [166]. By using an EpCAM independent blood filtration system, the ISET (isolation by size of epithelial tumour cells) and immunofluorescence it was recently 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 [77]. Another single-center prospective study that investigated whether CTCs are detectable in patients with previously untreated, stage III or IV NSCLC and whether their detection could provide prognostic information and/or early indication of patient response to conventional therapy, came to the conclusion that CTCs are detectable in these patients and constitute a novel prognostic factor for this disease [76].

21.1.3.2 Small-Cell Lung Cancer (SCLC)

The clinical significance and molecular characteristics of CTCs and CTC clusters, termed circu-

lating tumor microemboli (CTM), detected in patients with small-cell lung cancer (SCLC) undergoing standard treatment was evaluated. According to the results presented by Hou et al., both baseline CTC number and change in CTC number after one cycle of chemotherapy are independent prognostic factors for SCLC [56]

21.1.3.3 CTC as Surrogate Markers for Treatment Response in Lung Cancer

The group of Haber showed for the first time that lung cancer patients who's CTCs carried *EGFR* mutation known to cause drug resistance had faster disease progression than CTCs who lacked the mutation [89]. In late stage lung cancer patients *EGFR* mutations have been evaluated in single tumor cells enriched from blood using laser cell microdissection. In patients with advanced NSCLC mutational analysis with a 6-gene mutation panel *(EGFR, KRAS, BRAF, NRAS, AKT1,* and *PIK3CA)* were tested, where only one *EGFR* mutation (exon 19 deletion) was detected in CTC-derived DNA from the 38 patient samples analyzed [121].

The diagnostic test for ALK rearrangement in NSCLC for crizotinib treatment is currently done on tumor biopsies or fine-needle aspirations. Recently a group from the Institut de Cance'rologie Gustave Roussy attempted to avoid the need for a tissue sample to diagnose ALK-rearranged NSCLC by studying a novel ALK FISH method in CTCs. Pailler et al. recently detected ALK rearrangements 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 [106].

CellSearch™ technology was very recently adapted for the identification of tumor cells in Pleural effusions (PE) to assist in the diagnosis of malignant PEs. The pleural CellSearch™ assay may serve as a valuable addition to traditional cytology and provide useful information regarding the diagnosis of malignant effusions. Major advantages include that it is well standardized, relatively inexpensive, has a rapid turnaround, and is easily available [138].

21.1.4 Colorectal Cancer

In the official website of the National Institutes of Health (http://clinicaltrials.gov/ct2/home) our search on May 2014 on clinical studies, based on the key word "Circulating Tumor Cells AND colorectal cancer" revealed 57 studies (Fig. 21.2). The prognostic value of CTC and DTC 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 [48].

A comprehensive literature search was used to identify studies reporting on the significance of CTCs in the postoperative blood of CRC patients. Based on this search, a systematic review examined the evidence for the use of CTCs as prognostic markers in CRP. In six out of nine studies examined the detection of postoperative CTCs was found to be an independent predictor of cancer recurrence [111].

21.1.4.1 Metastatic Colorectal Cancer

In a prospective multicenter study, CTC were enumerated in 430 patients with mCRC at baseline and after starting first-, second-, or third-line therapy by using the CellSearch system. According to this study, the number of CTC 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 [20]. It was further shown that CTC enumeration before and during treatment independently predicts PFS and OS in advanced colorectal cancer patients treated with chemotherapy plus targeted agents and provides additional information to CT imaging [153]. 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 [91].

Another study showed a strong correlation between CTC detection and radiographic disease progression in patients receiving chemotherapy for colorectal cancer [27]. Evaluation of the impact of immediate and differing surgical interventions on CTC and their compartmentalization or localization in different anatomic vascular sites has shown that surgical resection of metastases, but not radiofrequency ablation, immediately decreases CTC levels [65]. Another recent study has shown that the qualitative and quantitative detection of CTC is higher in the mesenteric venous blood compartments of patients with CRC [125].

Very recently, Barbazan et al. evaluated the clinical utility of six CTC markers (tissue specific and Epithelial to Mesenchymal Transition transcripts) both as prognostic and predictive tools in mCRC patients. CTC-markers identified therapy-refractory patients not detected by standard image techniques. Patients with increased CTC-markers along treatment, but classified as responders by computed tomography, showed significantly shorter survival times [8].

In another recent study, post-chemotherapeutic CTCs were detected in stage III colon cancer patients in order to identify those patients who were at high risk of relapse. By using human telomerase reverse transcriptase *(hTERT), CK-19, CK-20,* and *CEA,* as the biomarkers to detect CTCs in 90 stage III colon cancer patients undergoing curative resection followed by mFOLFOX chemotherapy Lu et al. came to the conclusion that CTCs were independent predictors of post-chemotherapeutic relapse and that the persistent presence of post-chemotherapeutic CTCs in peripheral blood strongly correlated with reduced DFS and OS. The accuracy of detecting relapse in post-chemotherapeutic stage III colon cancer patients by analyzing the persistent presence of postchemotherapeutic CTCs was higher than that by postchemotherapeutic *CEA* levels chemotherapy [87].

21.1.4.2 Non-Metastatic Colorectal Cancer

The prognostic role of CTC in non-metastatic colorectal cancer is less clear than in mCRC. The low abundance of CTC in non-metastatic colorectal cancer requires very sensitive and specific detection methods. A recent review examined the possible clinical significance of CTC in non-metastatic colorectal cancer (TNM-stage I-III) with the primary focus on detection methods and prognosis. According to the findings reported, the presence of CTC in peripheral blood is a potential marker of poor disease-free survival in patients with non-metastatic colorectal cancer [150]. CTC detection might help in the selection of high-risk stage II colorectal cancer patient candidates for adjuvant chemotherapy, after enumerating CTC with the FDA-cleared CellSearch system [38].

Using *CEA, CK* and *CD133* as genetic markers, Iinuma et al. evaluated the clinical significance of CTCs as a prognostic factor for OS and DFS in the peripheral blood of patients with colorectal cancer who had undergone curative surgery. In the training sets, OS and DFS of patients who were positive for these markers were significantly worse than those of patients who were negative for these markers. At each staging analysis, OS and DFS of patients with Dukes' stage B or C cancer who were positive for *CEA/CK/CD133* were significantly worse than those of patients who were negative for these markers. In contrast, in patients with Dukes' stage A, no significant differences were seen between patients who were positive for these markers and those who were negative while in patients with Dukes' stage B and C cancer, *CEA/CK/CD133* demonstrated significant prognostic value. In validation sets, similar results were confirmed in patients with Dukes' stage B and C cancer. According to these data, in patients with Dukes' stage B and C CRC who require adjuvant chemotherapy, detection of *CEA/CK/CD133* mRNA in PB is a useful tool for determining which patients are at high risk for recurrence and poor prognosis [61]

21.1.4.3 CTC as Surrogate Markers for Treatment Response in Colorectal Cancer

The presence of *KRAS* and *BRAF* mutations reflect anti-EGFR therapy efficacy in metastatic colorectal cancer, and for this reason, primary tumors are analyzed for the presence of these specific mutations. However, discordances in

respect to the mutation status of *KRAS* and *BRAF* in metastatic colorectal cancer patients between primary tumors, CTC and metastatic tumors have very important implications [97]. There is a lot of work being done towards this direction; using the CellSearch system, Gasch C et al. investigated EGFR expression, *EGFR* gene amplification and *KRAS, BRAF* and *PIK3CA* mutations in single CTC of patients with metastatic colorectal cancer [37]. When *KRAS* mutations were detected in single CTC isolated from metastatic colorectal cancer patients a mutational concordance between CTCs and primary tumor in 50 % of matched cases was reported [32]. *APC, KRAS,* and *PIK3CA* mutations that were found in CTCs were also present at subclonal levels in the primary tumors and metastases from the same patient [52]. *KRAS* mutation status was also examined in CTC of metastatic colorectal cancer patients [160].

Plastin3 is a novel marker for CTC undergoing EMT and is associated with colorectal cancer prognosis that was particularly strong in patients with Dukes B and Dukes C [161]. Patients with CTC positivity at baseline had a significant shorter median PFS compared with patients with no CTCs and a significant correlation was also founded between CTC detection during treatment and radiographic findings at the 6 month staging [27].

CTCs are promising markers for the evaluation and prediction of treatment responses in rectal cancer patients, superior to the conventional tumor marker *CEA*. When the clinical significance of CTCs in comparison to CEA was investigated in respect to prediction of treatment responses there was a close relationship between CTC levels and treatment outcomes but serum *CEA* did not have any correlation [145, 146].

21.1.5 Melanoma

In the official website of NIH our search on May 2014 based on the key words "Circulating Tumor Cells AND melanoma" revealed 29 studies (Fig. 21.2).

CTC have been detected in peripheral blood of patients with metastatic melanoma and are associated with advanced melanoma stage and poor patient outcome. When the expression of *MART-1, MAGE-A3,* and *PAX3* mRNA has been evaluated in CTC of stage IV melanoma patients by RT-qPCR 54 % of patients were positive and the presence of CTC was significantly associated with DFS and OS [54, 55]. Kiyohara E et al. have recently developed a multimarker quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) assay for detecting CTC directly from peripheral blood specimens without the need of separating CTC from leukocytes. This assay, that is based on four mRNA biomarkers *(MART-1/Melan-A, MAGE-A3,PAX3,* and *GalNAc-T)* has both high sensitivity and specificity for CTC in blood specimens, and its clinical significance for serial bleed assessment of CTC in clinical trials and for daily clinical usage has been evaluated [72]

Chiu CG et al. very recently provided the first detailed genome-wide copy-number aberration (CNA) and loss of heterozygosity (LOH)-based characterization of melanoma CTC and illustrated how CTC may be used as a novel approach for identification of systemic metastasis. They characterized 251 CNA in CTC and their comparative analysis demonstrated >90 % concordance in SNP profiles between paired CTC and tumor metastases. In particular, there were notable recurring CNA across patients. In exploratory studies, the presence of several top CTC-associated CNA was verified in distant metastasis (stage IV) suggesting that certain genomic changes are propagated from regional metastases to CTC and to distant systemic metastases [19]. Uveal melanoma is one of the most deadly diseases in ophthalmology for which markers able to predict the appearance of metastasis are needed. A recent study that investigated the role of CTC as a prognostic factor in this disease confirmed the role of CTC as a negative prognostic marker in uveal melanoma patients after a long follow-up period. Further characterization of CTC will help understanding metastasis mechanisms in uveal melanoma and even improve patient management [92].

21.1.5.1 Early Stage Melanoma

CTC analysis may be useful in discriminating melanoma patients who may benefit from aggressive adjuvant therapy or stratifying patients for adjuvant clinical trials. The outcomes of patients with melanoma who have sentinel lymph node (SLN) metastases can be highly variable, which has precluded establishment of consensus regarding treatment of the group. The detection of high-risk patients from this clinical setting may be helpful for determination of both prognosis and management. Hoshimoto S et al. evaluated the clinical utility of a multimarker RT-qPCR *(MART-1, MAGE-A3,* and *GalNAc-T)* assay for the detection of CTCs in 331 patients with melanoma diagnosed with SLN metastases that were clinically disease-free after complete lymphadenectomy in a phase III, international, multicenter clinical trial. Individual CTC biomarker detection ranged from 13.4 % to 17.5 % and there was no association of CTC status with known clinical or pathologic prognostic variables. However, the presence of two or more positive biomarkers was significantly associated with worse distant metastasis, DFS and reduced recurrence-free survival [54, 55]

Blood-based assays to detect melanoma progression by monitoring levels of CTC and cfDNA can be used to evaluate progress and therapy response in melanoma patients [147] while advances in the molecular analysis of CTC may provide insight into new avenues of approaching therapeutic options that would benefit personalized melanoma management [73]. Mutated BRAF was detected in 81 % of 21 assessed stage IV melanoma patients [71]. When single, isolated CTC from patients with melanoma have been subjected to *BRAF* and KIT mutational analysis, the *BRAF* sequences and KIT sequences identified in CTC were inconsistent with those identified in autologous melanoma tumours, showing clonal heterogeneity [132].

21.1.6 Hepatocellular Carcinoma

In the official NIH website our search (May 2014) on clinical studies, based on the key word "Circulating Tumor Cells AND hepatocellular cancer" revealed 20 studies (Fig. 21.2).

The clinical relevance of CTC in hepatocellular carcinoma (HCC) is lagging behind other major tumor types. Up to now there are just a few studies on CTCs and hepatocellular carcinoma but this list is continuously growing. 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 CTC in HCC patients [164, 165]. Very recently, a remarkable variation of cells with epithelial, mesenchymal, liver-specific, and mixed characteristics and different size ranges were identifiable in the peripheral blood of HCC patients and the distribution of these cell subgroups varied significantly between different patient groups and was associated with therapeutic outcome [102]. By using the FDA cleared CellSearch™ system Schulze et al. investigated the prognostic relevance of EpCAM-positive CTCs in 59 patients with HCC and demonstrated a frequent presence of EpCAM-positive CTC in patients with intermediate or advanced HCC. The prognostic value of CTC detection in these cases for OS could have possible implications for future treatment stratification [137]. When the prognostic significance and the stem cell-like characteristics of EpCAM+ CTCs were identified prospectively in HCC patients undergoing curative resection, stem cell-like phenotypes were observed in EpCAM+ CTCs, and a preoperative CTC number of >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 <400 ng/mL or low tumor recurrence risk [145, 146].

21.1.7 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 CTC in peripheral blood may be a promising biomarker for the detection and prognosis of pancreatic cancer. In the official website of NIH our search on May 2014 based on the key word "Circulating Tumor Cells AND pancreatic cancer" revealed 14 studies (Fig. 21.2).

Tjensvoll et al., in a very recent review of previously reported studies on the clinical relevance of CTC detection in pancreatic cancer report that there is evidence that the presence of CTCs correlates with an unfavorable outcome [152]. 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 CellSeach system. CTC positivity was associated with poor tumor differentiation and with shorter OS in multivariable analysis [12, 13]. However, as stated by Gall et al., with such low numbers of CTCs detected in LAPC patients, it is unclear whether CTCs can actually contribute toward tumor invasiveness and spread in such an aggressive cancer. Although this is a well- designed study, the small number of patients with detectable CTCs means that the statistical power is not great enough to make firm conclusions. Therefore, this expensive assay needs further investigation before being used a prognostic marker in patients with LAPC [36]

A very recent meta-analysis aimed to assess the prognostic value of CTC 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 [50]. 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.

21.1.8 Gastrointestinal Cancers

The clinical significance of CTC detection in gastrointestinal (GI) cancer remains controversial and the molecular biological characteristics of CTCs are poorly understood. In the official NIH website our search (May 2014) based on the key word "Circulating Tumor Cells AND Gastointestinal Cancers" revealed 19 studies (Fig. 21.2). In a recent study, a total of 87 patients with metastatic or recurrent GI cancer were prospectively enrolled. CTCs and their *HER2* status were assessed using the CellSearch System. The findings of this study suggest that it is critical to evaluate the *HER2* status of not only the primary tumour but also the CTCs because the metastasizing tumour cells are the primary target of systemic therapy [62].

21.1.9 Head and Neck

In the official website of NIH our search on May 2014 based on the key word "Circulating Tumor Cells AND head and neck cancer" revealed 15 studies (Fig. 21.2).

According to a prospective clinical follow-up study of patients with squamous cell carcinoma of head and neck (SCCHN) undergoing surgical intervention, patients with no detectable CTCs had a significantly higher probability of DFS [63]. The same group has shown recently, that in patients with SCCHN, the presence of CTCs correlates with worse disease-free survival [7]. This conclusion was based on results obtained after isolation of CTC by a purely negative enrichment methodology which does not depend on the expression of surface epithelial markers. According to another prospective multi-centric analysis that studied the possible role of CTC identification in locally advanced head and neck cancer (LAHNC), CTC were frequently identified in oro- and hypopharyngeal cancer and in sinonasal undifferentiated carcinoma, SNUC; A decrease in the CTC number or their absence throughout the treatment seems also to be related with non-progressive disease, after both complete or

incomplete remission and with the proportion of patients alive and no evidence of disease [15, 159].

Current staging methods for squamous cell carcinomas (SCC) of the oral cavity (OSCC) need to be improved to predict the risk of individual patients. Grobe A et al. very recently assessed the prognostic significance of disseminated tumor cells (DTC) in bone marrow and CTC in peripheral blood from patients with OSCC. According to their findings both DTCs and CTCs are independent prognostic markers in patients with OSCC, predicting relapse with higher sensitivity at various disease stages than routine staging procedures [47].

21.1.10 Ovarian Cancer

In the official website of the NIH, our search (May 2014) based on the key word "Circulating Tumor Cells AND ovarian cancer" revealed 12 studies (Fig. 21.2).

Obermayr et al. identified a panel of six genes for the PCR-based detection of CTC in endometrial, cervical, and ovarian cancers and reported that by using this panel, they could detect 44 % of the cervical, 64 % of the endometrial and 19 % of the ovarian cancer patients [104]. 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 CTC in 24.5 % of the baseline (before primary treatment) and 20.4 % of the follow-up samples (6 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, sub-optimal debulking, and elevated CA-125 and HE-4 levels, whereas CTC during follow-up occurred more often in older and platinum resistant patients. PPIC positive CTCs during follow-up were significantly more often detected in the platinum resistant than in the platinum sensitive patient group, and indicated poor outcome independent from classical prognostic parameters [103].

By using the AdnaTest Breast Cancer commercially available test (Allere, USA) that is based on immunomagnetic enrichment and multiplex RT-PCR for selection and detection of CTCs Aktas et al., checked for CTC in the blood of 122 ovarian cancer patients at primary diagnosis and/or after platinum-based chemotherapy. They report that CTC positivity significantly correlated with shorter OS before surgery ($P=0.0054$) and after chemotherapy ($P=0.047$) [1]. 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 vs. PLD for relapsed ovarian cancer, by using the CellSearch system and reagents (Veridex). Results from this study indicated that elevated numbers of CTCs impart an unfavorable prognosis for ovarian cancer patients [119]. 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 [84].

21.1.11 Bladder Cancer

In the official website of NIH our search on May 2014 based on the key word "Circulating Tumor Cells AND bladder cancer" revealed 12 studies (Fig. 21.2). Nonmuscle-invasive bladder cancer is a tumor type characterized by early progression and a lack of prognostic markers and in this way it represents an optimal model to evaluate whether CTC assessment would be more beneficial in early stage cancer. Very recently, Raimondi C et al. reviewed whether CTCs may be used as a noninvasive, real-time tool for the stratification of early stage bladder cancer patients according to individual risk of progression [126].

Rink et al. prospectively detected and evaluated the biological significance of CTC in patients with bladder cancer, especially in those patients with non-metastatic, advanced bladder cancer

using the CellSearch. Their findings suggest that the presence of CTC may be predictive for early systemic disease since CTCs were detected in 30 % of patients with non-metastatic disease [130]. Gradilone et al., have chosen to evaluate the prognostic significance of survivin-expressing CTC in patients with T1G3 bladder tumours since the prognosis of T1G3 bladder cancer is highly variable and unpredictable from clinical and pathological prognostic factors. They report that the presence of CTC was an independent prognostic factor for DFS in patients with T1G3 bladder cancer [45]. CTCs have also 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 and came to the conclusion that CTC status is not likely to be a clinically useful parameter for directing therapeutic decisions in these patients [49].

21.1.12 Testicular Germ Cell Tumors

Germ cell tumors (GCTs) represent the most frequent malignancies among young men, but little is known about CTCs in these tumors. Nastaly et al., recently investigated the presence of CTCs in this tumor type, using two independent assays that target germ and epithelial cell-specific markers. For CTC detection, a combination of germ (anti-SALL4, anti-OCT3/4) and epithelial cell-specific (antikeratin, anti-EpCAM) antibodies was used because of the high heterogeneity of CTCs. Their results were correlated with disease stage, histology, and serum tumor markers. According to their findings, the inclusion of germ-cell specific markers improves CTCs detection in GCTs. CTCs occur frequently in patients with more aggressive disease, and there is a gradient of CTCs with decreasing numbers from the tumor-draining vein to the PB vessels [101].

21.1.13 Neuroendocrine Tumors

A recent single-center prospective study, aimed to determine the prognostic significance of CTCs in 176 patients with measurable metastatic neuroendocrine tumors (NETs). CTCs were measured using a semi-automated technique based on immune-magnetic separation of epithelial cell adhesion molecule-expressing cells. The presence of CTCs was associated with increased burden, increased tumor grade, and elevated serum chromogranin A (CgA). The presence of >one CTC was associated with worse PFS and OS; in multivariate analysis, CTCs remained significant when other prognostic markers, grade, tumor burden, and CgA were included. CTCs are a promising prognostic marker for patients with NETs and should be assessed in the context of clinical trials with defined tumor subtypes and therapy [69]

21.2 Quality Control Issues: Comparison of Different Methodologies

21.2.1 Analytical Methodologies for CTC Detection, Enumeration and Molecular Characterization

Since the detection of CTC has been shown to be of considerable utility in the clinical management of patients with solid cancers, a plethora of analytical systems for their isolation and detection have been developed and are still under development and their number is increasing at an exponential rate [80, 107–109, 163]. Since CTCs are very rare (1 CTC in 10^6–10^7 leukocytes) [151], in most cases they are specifically detected by using a combination of two steps: (a) isolation-enrichment and (b) detection. The only US Food and Drug Administration-cleared, commercially available CTC detection system is the CellSearch™ CTC test (Veridex, Raritan, NJ), which enriches CTCs by using particles that are coated with antibodies against EpCAM and is approved as a prognostic test in breast, colon, and prostate cancers.

The detailed presentation of these systems is beyond the scope of this review, especially since excellent reviews have been recently published on this topic (Pantel et al. 2012; Lianidou et al. 2011; [109, 163]; Alix-Panabieres et al. 2013).

21.2.2 Comparison Studies between Different CTC Assays

Advanced technologies developed for CTC isolation and detection are very promising for providing assays useful in oncological drug development, monitoring the course of disease in cancer patients, and in understanding the biology of cancer progression. However, the phenotypic heterogeneity of CTC and their low numbers in the bloodstream of patients, together with differences in pre-analytical sample processing, has led to the collection and accumulation of inconsistent data among independent studies [109]. Therefore, comparison of different methods for CTC enumeration and characterization by using the same samples is an important issue for the clinical use of CTC analysis as a liquid biopsy. However, as Powell et al. have recently shown, by performing a high dimensional single CTC profiling, CTC even within the ame patient are highly heterogeneous [120]. This heterogeneity of CTCs and their low numbers in the bloodstream of patients means that no standardized detection method currently exists. This, together with differences in pre-analytical sample processing, has led to the collection and accumulation of inconsistent data among independent studies.

We summarize here a number of recent studies that have focused on the comparison of different CTC methodologies, using the same clinical samples.

Andreopoulou et al. compared the CellSearch system and a molecular assay, the AdnaTest BreastCancer Select/Detect, to evaluate the extent that these assays differ in their ability to detect CTCs in the PB of MBC patients. The overall positive agreement between these two different methodologies was 73 % for CTC > 2 and 69 % for CTC > 5. These preliminary data suggest that theAdnaTest has equivalent sensitivity to that of the CellSearch system in detecting 2 or more CTCs. While there is concordance between these 2 methods, the AdnaTest complements the CellSearch system by improving the overall CTC detection rate and permitting the assessment of genomic markers in CTCs [4].

Khoja L et al. compared prospectively the utility of two platforms for CTC enumeration and characterisation in pancreatic cancer patients in a pilot exploratory study. Blood samples were obtained prospectively from 54 consenting patients and analysed by CellSearch and isolation by size of epithelial tumour cells (ISET). CellSearch exploits immunomagnetic capture of CTCs-expressing epithelial markers, whereas ISET is a marker independent, blood filtration device. CTC expression of epithelial and mesenchymal markers was assessed to explore any discrepancy in CTC number between the two platforms. According to their findings, ISET detects more CTCs than CellSearch and offers flexible CTC characterisation with potential to investigate CTC biology and develop biomarkers for pancreatic cancer patient management [70]

When three different CTC molecular assays were compared, using the same cDNAs throughout our study to avoid discrepancies due to pre-analytical errors all CTC assays gave similar results in about 70 % of cases. Better agreement was found in the metastatic setting, possibly explained by the higher tumor load in this group. Discordances could be attributed to the different gene transcripts used to evaluate CTC positivity. These results indicate the importance of CTC heterogeneity for their detection by different analytical methodologies [143].

The DETECT trial for metastatic breast cancer patients was designed to directly compare the prognostic impact of two commercially available CTC assays that are prominent representatives of immunocytochemical and RT-PCR based technologies. CTCs were assessed using both the AdnaTest Breast Cancer and the CellSearch system according to the manufacturers' instructions using 254 metastatic breast cancer patients. According to this study, when using the CellSearch system, there was a prognostic impact for OS even in the subgroups of patients with triple negative, HER2-positive and hormone receptor-positive/HER2-negative primary tumors while CTC-positivity assessed by the AdnaTest Breast had no association with PFS or OS. [98]

Gervasoni et al. compared the ability of three different methods to detect CTCs in the blood of colorectal cancer patients. Specifically, different aliquots of the same blood sample were screened for the presence of CTCs by a multimarker RT-PCR assay, the standardized CellSearch assay

and dHPLC-based gene mutation analysis. In the population tested, none of the blood samples analysed appeared to be positive by all three methods. The samples which were positive for CTCs by the CellSearch assay did not overlap with those that were positive by dHPLC. Interestingly, however, all of these samples were positive when assessed by RT-PCR. Conversely, of the samples that resulted negative by RT-PCR analysis, none appeared to be positive by either of the other methods. These data, therefore, indicate that of the three methods tested, the multimarker RT-PCR assay provides maximal probability of CTC detection [40].

When CTCs were compared with classic serum tumor biomarkers (*CA 15–3, CEA* and lactate dehydrogenase) as prognostic markers in metastatic breast cancer, it was found that elevated CTCs before cycle 2 are an early predictive marker of poor PFS and OS, which could be used to monitor treatment benefit [117].

21.2.3 Quality Control Issues

Standardization of CTC detection and characterization methodologies is important for the incorporation of CTC into prospective clinical trials testing their clinical utility. Despite the attractiveness and potential convenience of using blood-based CTC assays to diagnose genomic alterations and follow response to therapy in solid cancers, these technologies face significant hurdles and have not been included as yet in the guidelines to supplement tissue-based diagnostics. The main issues with CTC assays are the lack of standardized methods to define and capture these cells and the technical challenges in capturing a few CTC among billions of non-cancerous circulating blood cells.

Critical issues concerning the standardized detection of CTC include: (a) the standardization of the pre-analytical phase such as sampling itself (eg sample volume, avoidance of epidermal epithelial cells co-sampling in case that epithelial markers such as CK-19 will be later used for CTC detection), sample shipping (stability of CTC under different conditions) and storage conditions (use of preservatives, or anticoagulants), (b) standardization of CTC isolation through use of spiking controls in peripheral blood, and (c) standardization of detection systems (d) inter-laboratory and intra laboratory comparison studies for the same samples. The development of international standards for CTC enumeration and characterization is also very important especially in imaging detection systems that are observer-dependent (Lianidou 2011; Parkinson 2012).

Kraan et al. evaluated the feasibility of performing an external quality assurance (EQA) of the entire CellSearch procedure from blood draw to interpretation of results in multiple laboratories. Blood samples from six cancer patients and controls were distributed to 14 independent laboratories to test between-laboratory, between-assay, and between-instrument variation. Additionally, between-operator variability was assessed through the interpretation of blinded images of all blood samples on a website. According to the results of this study, shipment and storage of samples had no influence on CTC values. Between-instrument and between-assay variation was low indicating high reproducibility. However, between-laboratory CV ranged from 45 to 64 %. Although inter-operator agreement on image interpretation (Fleiss' κ statistics) ranged from "substantial" to "almost perfect," image interpretation, particularly of samples containing high numbers of apoptotic cells, was the main contributor to between-laboratory variation. This multicenter study has shown the feasibility of an EQA program for CTC detection in patient samples, and the importance of continuation of such a program for the harmonization of CTC enumeration [75].

A very recent study evaluated the inter-reader agreement of the results obtained with the FDA-cleared CellSearch sytem for HER-2 in breast cancer, using exactly the same CTC images. For this reason, the same CellSearch images were sent to 22 readers from 15 academic laboratories and 8 readers from two Veridex laboratories. The inter-reader agreement for CTC definition was high, while reduced agreement was observed

in M0 patients with low CTC counts. Continuous training and independent image review are required [60].

A recent manuscript summarized in a global aspect current thinking on the value and promise of evolving CTC technologies for cancer patient diagnosis, prognosis, and response to therapy, as well as accelerating oncologic drug development. According to Parkinson et al., moving forward requires the application of the classic steps in biomarker development-analytical and clinical validation and clinical qualification for specific contexts of use [109]. There is still a lot to be done for the automation, standardization, quality control and accreditation of analytical methodologies used for CTC isolation, detection and molecular characterization. When this goal is achieved, the next logical step will be to use CTC technologies to diagnose patients, select biomarker-based therapeutics, and monitor response to therapies using not only pathologic tissues but also CTCs.

21.3 Conclusions: Future Perpectives

The main advantage of CTC analysis is based on their unique potential to offer a minimally invasive "liquid biopsy" sample, easily obtainable at multiple time points during disease history which 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 [3, 80].

CTC downstream molecular characterization at the protein, DNA [16, 17] and RNA level, could now serve as a "liquid biopsy" approach and eventually offer additional information and even more a serious advantage over the conventional and well established tumor biopsy approach since peripheral blood samples can be frequently and sequentially obtained [3, 80].

Cell free DNA (cfDNA) circulating in plasma or serum of cancer patients has also been recently proposed as an alternative to CTCsliquid biopsy approach [11, 30, 90]. It has been recently shown that by using extremely powerful and highly sensitive detection techniques, the presence of specific mutations in plasma of cancer patients could give valuable information concerning response to specific molecular targeted therapies [99]. However, there is a substantial difference between these two approaches; CTCs are viable cells, circulating in blood, and understanding their biology in a holistic way, could give valuable information on the metastatic spread, elucidate their connection to cancer stem cells, and reveal active and possible targetable signalling networks, while cfDNA can give specific information as a circulating biomarker, for the presence or absence of specific alterations indicating therapy response.

Co-development of anticancer therapeutics with CTC-based diagnostics could enable clinical validation and qualification of CTC-based assays as companion diagnostics in the near future [123]. Further research on the molecular characterization of CTC will provide important information for the identification of therapeutic targets and understanding resistance to therapies. The molecular characterization of CTC is highly challenging especially in combination with next generation sequencing technologies that will enable the elucidation of molecular pathways in CTC and will probably lead to the design of novel molecular therapies targeting specifically CTC. Even if this is still far from being considered to be applied in a routine clinical setting, it holds a great promise for the future management of cancer patients.

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