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.

Introduction

The presence of tumor cells circulating in the blood of cancer patients was first reported by Thomas Ashworth in 1869. Almost 150 years after this first report, the importance of circulating tumor cells (CTCs) detection and molecular characterization is becoming evident. 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. CTC detection 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. New areas of research are directed towards developing novel assays for CTC molecular characterization. High heterogeneity of CTCs, even among the same individuals, has been observed when performing high-dimensional single CTC profiling, and by

Keywords

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

History

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directly measuring gene expression in individual CTCs without the common practice of pooling such cells\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{12}.

**Metastatic breast cancer**

In their seminal paper many years ago, Cristofanilli and colleagues clearly showed, using the CellSearch System (Veris, 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{20}. By using a real-time RT-qPCR assay for CK-19 mRNA\textsuperscript{21,22}, CTC detection was shown to be an independent prognostic factor for reduced disease-free interval and overall survival before\textsuperscript{23}, during\textsuperscript{24}, and after\textsuperscript{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\textsuperscript{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 chemotherapy was associated with poorer clinical outcome in patients with locally advanced breast cancer\textsuperscript{27}. This finding is in agreement with the results of a recent study that showed that CTC enumeration at diagnosis, using the CellSearch assay, is a powerful predictor of poor clinical outcome in patients with triple-negative breast cancer\textsuperscript{28}.

**Colorectal cancer**

CTC enumeration: CellSearch (FDA cleared) CK-19, EpCAM, CA125, PDL-3, CEACAM5, KRAS mutations

**Breast cancer**

CTC enumeration: CellSearch (FDA cleared) CK-19, HER2, ER, PR, EpCAM, MUC1, EMT markers, StemCell markers, DNA methylation

**Ovarian cancer**

EpCAM, MUC-1, HER-2, PPIC, CCNE2, DSKFZp762E1312, EMP2, MAL2, SLC6A8

**Hepatocellular carcinoma**

Cytokeratins, EpCAM, ASGP1, N-cadherin, vimentin

**Lung cancer**

EpCAM, CK-19, CK-7, MUC1, vimentin, cadherins nTERT, TTF-1, EGFR mutations, ALK rearrangements

**Pancreatic cancer**

CTC enumeration: CellSearch (CK, CD45)

**Bladder cancer**

CTC enumeration: CellSearch CK-8, survivin

**Prostate cancer**

CTC enumeration: CellSearch (FDA cleared) PSA, KLK2, AR, PSCA, ERG, AR and PTEN, AR mutations, MYC, BRCA1 allelic imbalances, TMPRSS2-ERG rearrangements

**Melanoma**

TYR, MUC-18, p97, MART-1, MAGE-A3, MLANA, MITF, GalNAc-T, BRAF mutations

**Head and neck**

CK, vimentin, EGFR, CD44, N-cadherin

**CTC: The liquid biopsy approach**

**Figure 1. CTC analysis and molecular characterization in various types of solid cancers.**
chemotherapy can accurately predict OS²⁷. 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 hormone-therapy-resistant residual disease²⁸. Lucci et al. prospectively collected data on CTCs at the time of definitive surgery from chemo naive 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 chemo naive patients with non-metastatic breast cancer²⁹.

**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 patients³⁰–³⁵. Based on this evidence, re-evaluation of the 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 tumor³⁶. Liglhart 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 patient³⁷. Many research groups have already shown that HER2-positive CTCs can be detected in patients with HER2-negative primary tumors³⁰,³²,³⁴,³⁸,³⁹. Georgoulas 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 patients⁴¹.

CTCs play a crucial role in metastasis, and epithelial-mesenchymal transition (EMT) is an essential process in the metastatic cascade⁴²,⁴³. A major proportion of CTCs of metastatic breast cancer patients show EMT and tumor stem cell characteristics⁴⁴. As well, CTCs expressing Twist and vimentin were identified in patients with metastatic and early breast cancer⁴⁵. 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 microscopy⁴⁶. Currently used detection methods for CTCs are not sufficient at identifying this subtype of CTCs that underwent EMT³⁷.

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 progression⁴⁸. The expression levels of EMT-inducing transcription factors have been determined in CTCs in primary breast cancer patients⁴⁹. 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 CTCs⁵⁰.

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 CTCs⁵¹. 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) samples⁵². 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 patients⁵³. 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
platinum-resistant than in the platinum-sensitive patient group, and indicated poor outcome independent of classical prognostic parameters.

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 ($p=0.047$). 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.

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.

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

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Detection method used</th>
<th>Biomarker used</th>
<th>Prognostic significance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary ovarian cancer</td>
<td>RT-qPCR</td>
<td>CCNE2, DKKFZ762E1312, EMP2, MAL2, PPI and SLC6A8</td>
<td>Not evaluated</td>
<td>53</td>
</tr>
<tr>
<td>Epithelial ovarian cancer</td>
<td>RT-qPCR</td>
<td>PPIC, EpCAM</td>
<td>OS: yes DFS: yes</td>
<td>54</td>
</tr>
<tr>
<td>Primary ovarian cancer</td>
<td>AdnaTest, RT-qPCR</td>
<td>EpCAM+, MUC-1, and HER2, CA 125</td>
<td>OS: yes PFS: no</td>
<td>55</td>
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<tr>
<td>Advanced ovarian cancer</td>
<td>CellSearch</td>
<td>Enumeration of CTCs</td>
<td>OS: yes DFS: yes</td>
<td>56</td>
</tr>
<tr>
<td>Newly diagnosed or recurrent</td>
<td>CellSearch</td>
<td>Enumeration of CTCs</td>
<td>OS: no DFS: no</td>
<td>57</td>
</tr>
<tr>
<td>epithelial ovarian cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

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. 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. 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. 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.

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. 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. The combination of CTC and PSA velocity may offer insights into the prognosis and management of advanced prostate cancer.

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. 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.

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. 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. 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. 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. 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.

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

BRCA1 allelic imbalances were also detected among CTCs in multifocal prostate cancer using FISH analysis. 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. 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.

Circulating endothelial cells, CTCs, and tissue factor levels alone and combined can predict OS early on in CRPC patients treated with docetaxel-based therapy. 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).

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. 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.

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. 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.

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. 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.

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

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Detection method used</th>
<th>Biomarker used</th>
<th>Prognostic significance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early NSCLC</td>
<td>FISH</td>
<td>ALK rearrangement, cytokeratins, E-cadherin, vimentin, N-cadherin</td>
<td>Not evaluated</td>
<td>85</td>
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<tr>
<td></td>
<td>RT-PCR</td>
<td>EpCAM/MUC1 mRNA</td>
<td>OS: yes DFS: yes</td>
<td>81</td>
</tr>
<tr>
<td>Advanced NSCLC</td>
<td>ISET, IF</td>
<td>CK, vimentin</td>
<td>OS: not evaluated PFS: not evaluated</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Allele-specific PCR</td>
<td>EGFR mutations</td>
<td>OS: not evaluated DFS: yes</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>CellSearch, RT-PCR, ARM5 real-time PCR</td>
<td>Enumeration of CTCs, EGFR expression, EGFR mutations</td>
<td>OS: N.V. DFS: yes</td>
<td>84</td>
</tr>
</tbody>
</table>
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. 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. 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. Another study showed a strong correlation between CTC detection and radiographic disease progression in patients receiving chemotherapy for colorectal cancer. 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. Another recent study showed that the qualitative and quantitative detection of CTCs is higher in the mesenteric venous blood compartments of patients with mCRC.

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. 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.

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. 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. 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. 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. KRAS mutation status was also examined in CTCs of metastatic colorectal cancer patients.

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. 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. Inuma 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.

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
results have been achieved in HCC CTC detection, although the knowledge of 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{109}. The same group has shown recently that in patients with SCCHN, the presence of CTCs correlates with worse disease-free survival\textsuperscript{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\textsuperscript{111}.

**Bladder cancer**

The finding of extravasal 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{115}. Advances in the molecular analysis of CTCs may provide insight into new avenues of approaching therapeutic options that would benefit personalized melanoma management\textsuperscript{116}. Mutated \textit{BRAF} was detected in 81% of 21 assessed stage IV melanoma patients\textsuperscript{117}. Single, isolated CTCs from patients with melanoma have been subjected to
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