Circulating Tumor Cells vs. ctDNA;
Complementary or Competitive Liquid Biopsy Approaches?
Introduction

Tumors are highly heterogeneous and sampling by traditional biopsy may be painful, invasive and precluded by tumor size or location. Liquid biopsies are being investigated as a method for minimally-invasive, systemic, assessment of tumors using a blood sample from the patient. Two of the most common liquid biopsy approaches rely on the quantification and analysis of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in the blood. The analysis of CTCs or ctDNA in blood samples is being studied as means for serial monitoring of epithelial cancers (e.g. breast, lung, colorectal, prostate), prediction of prognosis, tailoring and monitoring response to cancer therapy, and detailed biologic analysis, on an individual patient basis.

Both ctDNA and CTC are rare events, even in patients with advanced cancer, and their detection and analysis requires sensitive analytic approaches.1-5 Despite a paucity of studies directly comparing the two sources of tumor sample, ctDNA and CTC approaches to liquid biopsy have become competing biomarkers.6 However, the information obtained from ctDNA and CTCs is different, complementary and depends on the context of use.7, 8 As ctDNA and CTC technologies evolve, they will likely have overlapping as well as distinct clinical applications, based on their biologic and technologic strengths and weaknesses.

Circulating tumor DNA (ctDNA)

DNA is released into the bloodstream as fragments by lytic, apoptotic or necrotic cells, or by active secretion from macrophages following phagocytosis.7, 9-11 Although the majority of the released [extracellular] DNA is adsorbed to the surface of leukocytes or erythrocytes, a portion remains unbound and can be identified in the plasma, known as cell free DNA (cfDNA). The portion of cfDNA that is derived from tumor cells is called ctDNA. ctDNA has a short half-life in the circulation, ranging from 15 minutes to several hours.5,12 Although the total concentration of ctDNA in blood varies considerably patient-to-patient, ctDNA usually represents <0.1-10% of the total cfDNA in the circulation of late-stage cancer patients.6 ctDNA-based techniques analyze tumor heterogeneity at the level of genomic aberrations. Highly sensitive analytical platforms have been developed for the analysis of ctDNA, to detect single-nucleotide mutations or whole-genome sequencing to establish copy-number changes.13 Technologies can be divided into targeted approaches14-16 and untargeted approaches.17-19 Targeted approaches aim to detect known, characteristic genetic or epigenetic modifications in ctDNA.10 Untargeted approaches screen the genome and discover new genomic alterations.

In general, targeted approaches have a higher analytic sensitivity than untargeted approaches.20 The most sensitive methods have a lower limit to detect 1 mutated copy in a background of 10,000 wild-type copies.15

Although ctDNA analyses are capable of examining the genetic and epigenetic changes in tumor DNA (mutations, translocations, amplifications, insertions and deletions, and methylation abnormalities), potential exists for false-negative and false-positive results, given that ctDNA is not isolated distinct from cfDNA. In addition, by definition, ctDNA approaches cannot analyze the tumor RNA transcriptome, proteome, or allow morphological or functional study of the tumor. However, an advantage of ctDNA is that it can be analyzed from biobanked fluids, such as frozen plasma (Table 1).

Table 1. CTC and ctDNA Analysis Opportunities

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<td><strong>DNA aberrations</strong></td>
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<td>- point mutations</td>
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<td><strong>Epigenetic modifications</strong></td>
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<td>- methylation patterns</td>
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<td><strong>RNA aberrations</strong></td>
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<td>- microRNA</td>
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<td>- long noncoding RNA</td>
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<td>- translocations</td>
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<td>- xenotransplantation</td>
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<td><strong>Protein expression/localization</strong></td>
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<td><strong>Option to use biobanked samples</strong></td>
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<td>- frozen plasma</td>
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<td>- urine, other biofluids</td>
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Circulating tumor cells (CTCs)

Circulating tumor cells (CTCs) are released into the bloodstream from the primary tumor and/or metastatic sites. CTCs are frequently present in the blood of patients with breast, lung, prostate and colon cancers, and have been associated with decreased progression-free and overall survival in patients.\(^{21-24}\) CTCs are typically present at concentrations as low as 1-10 CTCs per 100 million to 1 billion blood cells in cancer patients.\(^{25-27}\) The half-life of CTCs in the bloodstream is estimated to be only 1-2.4 hr,\(^{28}\) before clearance by extravasation into secondary organs.

In addition to the utility of counting CTCs in the blood for tumor monitoring and prognosis, isolation and analysis of CTCs has great potential to allow study of fundamental processes of tumor progression, response to therapy, metastasis and CTCs’ role in establishing metastatic disease. CTCs allow single-cell identification and characterization of tumor heterogeneity to elucidate biology at the DNA, RNA, and protein levels (Table 1). RNA-based approaches are advantageous given that each cell harbors thousands of copies of each RNA transcript, as opposed to only two copies of chromosomal DNA in each diploid cell.\(^{29}\)

Although functional studies can be challenging given the low numbers of CTCs isolated from patients’ samples, the development of sophisticated single-cell analysis technologies have allowed for new insights into the genetic make-up of CTCs and have demonstrated a marked inter- and intra-patient heterogeneity of CTCs.\(^{8}\) Sensitive deep-sequencing technologies have shown that mutations found in CTCs resemble those detected in both the primary tumor and metastases.\(^{30, 31}\) More detailed analysis of CTCs may reveal whether CTCs have a typical spectrum of alterations in their genome or proteome, whether they represent a dominant clone of their respective primary tumor, or whether different subclones are equally represented in the peripheral blood. Identification of mutations or dysregulated protein expression may help identify new therapeutic targets.

Given that viable tumor cells drive cancer progression and cause therapy resistance, the study of viable CTCs offers vast opportunity to study the biology of epithelial tumors using multiple approaches. However, the rarity of CTCs remains a major challenge. A high recovery rate without compromising on purity and viability are key requirements for capture techniques.\(^{26,32}\)

Many current approaches capture CTCs based on expression of epithelial-specific markers. Expression of the epithelial cell adhesion molecule, EpCAM\(^{33}\), is required for the capture of CTCs using the only FDA-approved clinical CTC platform, CELLSEARCH® (Janssen Diagnostics, LLC, USA).\(^{34}\) Using the CELLSEARCH system, the prognostic utility of CTCs in breast, prostate and colon cancers has been demonstrated.\(^{34}\)

However, a key limitation to this approach is considerable cell loss (20-40%) caused by the inability to detect CTCs with low or absent EpCAM expression, such as those that have gone through or are undergoing epithelial-to-mesenchymal transition (EMT).\(^{35, 36}\) Cells with an intermediate phenotype, a partial EMT, may have the highest plasticity to adapt to the conditions present at metastatic sites.\(^{37}\) Thus, label-free CTC capture technologies that do not rely on particular surface markers to enrich for CTCs are appealing to allow capture and analysis of the full spectrum of heterogeneity among CTC populations found in the blood.

Complementary or Competitive:
Clinical applications and findings using CTC and ctDNA approaches

CTCs and ctDNA have been investigated for application in cancer screening/early detection, estimating prognosis/risk for metastatic relapse, stratification and real time monitoring of therapy, identification of therapeutic targets and resistance mechanisms. Table 2 summarizes key findings in each area. Many of these studies should be validated in larger cohorts, and sources that may lead to non-specific findings in non-cancer patients need to be identified.

To date, the number of patients included in ctDNA studies is much smaller than in CTC-based studies. Although many studies have shown utility for CTC or ctDNA individually, few studies have performed analyses of both CTC and ctDNA, especially using the most up-to-date technology and methods to allow fair comparison. However, several clinical trials which aim to quantify both CTC and ctDNA in a variety of tumors and treatment settings are underway, and may provide clearer evidence of their relative utility, strengths and weaknesses as tumor samples [ClinicalTrials.gov, NCT02771314, NCT02364557, NCT02735252, NCT02588105, NCT02269982, NCT02220556, NCT02370355].
### CTC ctDNA

#### Cancer screening/early detection

- • CTCs could be detected in 5 of 168 patients with COPD without clinically detectable lung cancer. Annual surveillance detected lung nodules 1-4 years after CTC detection, leading to diagnosis of early-stage lung cancer. No CTCs were detected in control subjects.38
- • Among 640 patients with various cancer types, ctDNA was identified in only 48-73% of patients with localized cancers [digital PCR].5
- • Somatic alterations in ctDNA were detected in the majority of patients with stage II-IV non-small cell lung cancer, but only 50% of patients with early-stage disease, using CAPP-Seq.39

#### Estimating prognosis/risk of relapse

- • In the largest multicenter study so far, the presence of CTCs was associated with poor disease-free survival, breast cancer-specific survival, and overall survival. CTCs were an independent prognostic marker in multivariable analysis. The persistence of CTCs after chemotherapy also showed a negative influence.40
- • High-risk prostate cancer patients with persistent CTCs post-radical prostatectomy developed biochemical recurrence.41
- • CTC counts and metastatic relapse have been correlated in other tumors, such as colorectal,42 bladder,43,44 liver45 and esophageal.46
- • DNA amplifications, identified in ctDNA of breast cancer patients, were detectable in some samples for up to 12 years after diagnosis, indicating the presence of “occult” micrometastatic disease.47
- • Of PIK3CA-mutant breast cancer patients, 23% were positive for a PIK3CA mutation that was a significant and independent prognostic factor.48
- • Relapse could be detected months earlier when ctDNA levels were quantified using droplet digital PCR, compared to conventional follow up, in colorectal cancer patients.49
- • CTCs can be used to track clonal evolution and resistance to targeted therapy of colorectal cancer.53,54
- • Mutations resulting in acquired resistance to targeted therapy for colorectal cancer are detected in ctDNA before progression can be documented by imaging.55-57
- • Increases in ctDNA mutation levels were associated with acquired drug resistance following treatment of metastatic breast cancer with paclitaxel, tamoxifen and trastuzumab, and lapatinib.19
- • AR gene aberrations (i.e. missense mutation, amplification) detected using ctDNA predict outcomes of antiandrogen therapy for prostate cancer.58-60

#### Monitoring therapies in real time

- • In an analysis of samples from 17 international centers, including 1944 patients from 20 studies, CTC counts improved the prognostication of metastatic breast cancer when added to full clinicopathologic predictive models, whereas tumor markers did not, despite their common clinical use.51
- • A biomarker panel containing CTC number and LDH level was a surrogate for survival in individual prostate cancer patients, whereas changes in serum PSA levels were not relevant.52
- • ctDNA can be used to track clonal evolution and resistance to targeted therapy of colorectal cancer.53,54
- • Mutations resulting in acquired resistance to targeted therapy for colorectal cancer are detected in ctDNA before progression can be documented by imaging.55-57
- • Increases in ctDNA mutation levels were associated with acquired drug resistance following treatment of metastatic breast cancer with paclitaxel, tamoxifen and trastuzumab, and lapatinib.19
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<td><em>Breast tumors classified as Estrogen Receptor (ER)-positive can have heterogeneous (ER+ and ER-) CTCs, which may escape endocrine therapy.</em>&lt;sup&gt;61&lt;/sup&gt;</td>
<td><em>ctDNA analysis can uncover genomic aberrations that may affect the efficacy of drugs that target their protein product (i.e. EGFR, KRAS, TP53, PIK3CA, AR mutations)</em>&lt;sup&gt;53-57, 78-80&lt;/sup&gt;</td>
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<td>A CTC-ETI (endocrine therapy index), which combines CTC enumeration and the expression of ER, BCL2, HER2 and Ki67, is being prospectively evaluated for its ability to predict resistance to endocrine therapy in patients with ER-positive metastatic breast cancer.&lt;sup&gt;62&lt;/sup&gt;</td>
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<td>Overt distant metastases and CTCs can have discrepant HER2 status compared to the primary tumor.&lt;sup&gt;63-65&lt;/sup&gt;</td>
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<td>PD-L1 is frequently expressed on CTCs (&gt;60% of patients) in patients with hormone-receptor-positive, HER2 negative breast cancer&lt;sup&gt;66&lt;/sup&gt;, and can be monitored in trials of patients undergoing immune checkpoint blockade.&lt;sup&gt;67&lt;/sup&gt;</td>
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<td>PSA/PSMA-based measurements are surrogates for androgen receptor (AR)-signaling in CTCs, and might help predict the outcome of AR-based therapy.&lt;sup&gt;35&lt;/sup&gt;</td>
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<td>mRNA expression of ARV7, a truncated form of AR that lacks the ligand-binding domain but remains constitutively active, in CTCs may predict resistance or sensitivity to anti-androgen therapy.&lt;sup&gt;68, 69&lt;/sup&gt; RNA sequencing of CTCs in metastatic castration resistant prostate cancer have revealed additional mechanisms of resistance.&lt;sup&gt;70&lt;/sup&gt;</td>
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<td>RNA-ISH demonstrated miRNA heterogeneity among CTCs in patients with breast, prostate or colorectal cancer.&lt;sup&gt;71&lt;/sup&gt;</td>
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<td>Mutations in genes encoding therapeutic targets or signaling proteins downstream of the target can affect the efficacy of targeted drugs, e.g. mutated EGFR in lung cancer&lt;sup&gt;72, 73&lt;/sup&gt; and KRAS in colorectal cancer, AR in prostate cancer&lt;sup&gt;74&lt;/sup&gt;, HER2 in breast cancer.&lt;sup&gt;75-77&lt;/sup&gt;</td>
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Capture of CTCs using the Celsee™ PREP Platform

Celsee Diagnostics’ integrated CTC detection platform, Celsee PREP, captures CTCs from 4-8 mL whole blood into individual compartments in a microfluidic slide based on size and deformability differences from blood cells. The microfluidic slide consists of 56,320 capture chambers that ensure each captured CTC is isolated in its own compartment, enabling single-cell, on-chip analysis, and limiting the potential for leukocyte contamination. In order to avoid cell loss caused by heterogeneous expression of cell surface proteins, the Celsee PREP platform uses a label-free approach to capture CTCs. Thus, a wide array of antibodies and DNA/RNA-based probes can be used for downstream identification and characterization of individual CTCs.

In analysis of normal human donor blood samples spiked with cancer cell lines (breast, prostate, colorectal), the Celsee platform could capture between 20-2,000 cells with a capturing efficiency of >85% and high reproducibility (CV, <7%), with minimal leukocyte contamination. Notably, the device captured both epithelial cancer cells (MCF7 and SKBR3) and mesenchymal cells (MDA-MB-231) with equal efficiency.

After capture of CTCs, counts, protein phenotype, and DNA FISH or mRNA FISH analysis can be performed, on-chip [Figure 1, top]. Circulating tumor cells are defined by the presence of positive nuclear [DAPI] and cytokeratin [Pan-CK] staining, and lack of staining for the leukocyte common antigen, CD45 [Pan-CK*DAPI*CD45*]. Contaminating WBCs can be differentiated from CTCs by their differential staining for cytokeratin and CD45. [Pan-CK-DAPI*CD45*]. Data can be collected manually by fluorescence microscopy, or automatically, using the Celsee ANALYZER imaging station. The Celsee ANALYZER automatically scans up to 8 microfluidic slides, capturing the images of captured CTCs, both in brightfield and in fluorescence (up to 4 colors). Cells of interest can be rapidly reviewed for their heterogeneity, cellular and genomic makeup. CTCs are identified, imaged and location cataloged. The software also allows the quantification of images, analysis and report generation.

Figure 1. Types of analysis that can be performed with CTCs retrieved by the Celsee PREP platform.
Alternatively, CTCs can be enriched and retrieved from the microfluidic chip for NGS or qRT-PCR analysis (Figure 1). Whole blood is depleted of RBCs and WBCs prior to capture on the Celsee PREP100 using RosetteSep CD45 and density gradient centrifugation. Following capture, CTCs are removed from the microfluidic chip by backflow for off-chip analyses (Figure 2). Studies have shown a 91% (range 76-100%) recovery rate of backflow CTC retrieval.

The Celsee PREP100 allows researchers to run individual samples, and customize experimental assays on isolated CTCs, reducing the time for discovery. The Celsee PREP400 and Celsee ANALYZER offer the advantage of automation, with integration of the processes of sample preparation, image analysis of CTCs and molecular characterization of the captured cells by immunostaining. Blood samples from 200 healthy donors and 128 patients with metastatic breast cancer (n=96), prostate cancer (n=27) and colorectal cancer (n=5) were processed using the automated Celsee PREP400 system. The Celsee PREP400 captured CTCs from each of the patient samples (range 1-2,457 CTC); no CTCs were detected in any of the healthy donor samples. Further, in a comparison of 18 samples from patients with metastatic prostate cancer, CTCs were detected in 61% (11/18) samples using the FDA-cleared CELLSEARCH system, compared to 94% of the samples (17/18) by the Celsee PREP system. CTC counts were significantly higher using the Celsee PREP system, suggesting greater sensitivity for CTC detection.

Summary

Liquid biopsy is being studied as means for serial monitoring of epithelial cancers (e.g. breast, lung, colorectal, prostate), prediction of prognosis, tailoring and monitoring response to cancer therapy, and detailed biologic analysis, on an individual patient basis. Both ctDNA and CTC are rare events, and their detection and analysis requires sensitive analytic approaches. ctDNA and CTC likely have overlapping as well as distinct clinical applications. The Celsee PREP platform uses a label-free approach to capture CTCs, and allows flexibility in downstream analysis and characterization of individual CTCs, on- or off-chip. The optional Celsee ANALYZER imaging station allows additional flexibility to the researcher by allowing automated data collection and analysis.

Figure 2. Whole blood is depleted of blood cells and a backflow performed to capture a highly-enriched CTC population.
References


