Circulating Tumor Cells: A Multifunctional Biomarker

Timothy A. Yap1,2, David Lorente1,2, Aurelius Omlin3, David Olmos4, and Johann S. de Bono1,2

Abstract

One of the most promising developments in translational cancer medicine has been the emergence of circulating tumor cells (CTC) as a minimally invasive multifunctional biomarker. CTCs in peripheral blood originate from solid tumors and are involved in the process of hematogenous metastatic spread to distant sites for the establishment of secondary foci of disease. The emergence of modern CTC technologies has enabled serial assessments to be undertaken at multiple time points along a patient’s cancer journey for pharmacodynamic (PD), prognostic, predictive, and intermediate endpoint biomarker studies. Despite the promise of CTCs as multifunctional biomarkers, there are still numerous challenges that hinder their incorporation into standard clinical practice. This review discusses the key technical aspects of CTC technologies, including the importance of assay validation and clinical qualification, and compares existing and novel CTC enrichment platforms. This article discusses the utility of CTCs as a multifunctional biomarker and focuses on the potential of CTCs as PD endpoints either directly via the molecular characterization of specific markers or indirectly through CTC enumeration. We also discuss issues relating to intrapatient heterogeneity and the challenges associated with isolating CTCs undergoing epithelial–mesenchymal transition, as well as apoptotic and small CTCs. Finally, we envision the future promise of CTCs for the selection and monitoring of antitumor precision therapies, including applications in single CTC phenotypic and genomic profiling and CTC-derived xenografts, and discuss the promises and limitations of such approaches.

See all articles in this CCR Focus section, “Progress in Pharmacodynamic Endpoints.”

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Disclosure of Potential Conflicts of Interest

T.A. Yap reports receiving speakers bureau honoraria from Janssen. A. Omlin is a consultant/advisory board member for Pfizer, Astellas, AstraZeneca, and Janssen. D. Olmos reports receiving speakers bureau honoraria from Veridex. J.S. de Bono reports receiving speakers bureau honoraria from Johnson & Johnson. No potential conflicts of interest were disclosed by the other authors.

CME Staff Planners’ Disclosures

The members of the planning committee have no real or apparent conflict of interest to disclose.

Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the strategies currently under investigation for the development of circulating tumor cells as a minimally invasive multifunctional biomarker in patients with a range of different cancers.

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Introduction

The advent of rationally designed molecular therapeutics that inhibit specific tumoral molecular aberrations has led to a paradigm shift in our understanding of cancer and drug discovery (1). In contrast with previous drug development strategies, there is now a general acceptance that maximum-tolerated doses may not necessarily correlate with the biologic efficacy of therapy, and therefore the need for molecular tools or biomarkers that can accurately assess the underlying mechanisms of action and pharmacodynamic (PD) effects of the drug has emerged (2).

Such PD biomarkers, together with pharmacokinetic (PK) parameters, provide confirmation that pharmacologic
effects of a novel antitumor compound on its intended target, pathway, and associated functions have taken place (3). Such PD biomarkers may be used to define the quantitative extent and duration of target inhibition required in the clinic for biologic and therapeutic effects, and ultimately in making "go" or "no-go" drug development decisions (1). Although indirect PD readouts such as mechanism-based toxicities, for example, rash with EGF receptor (EGFR) inhibitors, are useful general indicators of pharmacologic blockade, modern technologies now permit the direct quantification of protein phosphorylation knockdown or other related parameters in tumor biopsies. The recent development of minimally invasive PD assays may reduce the risks and issues associated with repeated tumor biopsies and enable serial determinations of drug effects, thus minimizing the impact of inter- and intrapatient variability on such results.

One of the most promising developments has involved the emergence of circulating tumor cells (CTC) as a minimally invasive biomarker in cancer medicine. This review focuses on the utility of CTCs as PD endpoints in the monitoring of novel molecularly targeted therapeutics. We also discuss the technical aspects of CTS assays and detail existing and novel CTC enrichment technologies. We propose strategies for incorporating CTCs as PD biomarkers into early-phase clinical trials, discuss the promises and limitations of CTCs, and envision the future utility of CTCs as multifunctional biomarkers in modern clinical studies.

CTCs: Emergence as a Multifunctional Biomarker

It is now widely accepted that CTCs found in peripheral blood originate from solid tumors and are involved in the process of hematogenous metastatic spread by shedding from such cancers and migrating to distant sites for the establishment of secondary foci of disease (4). CTCs are ultimately rare events, with a frequency of approximately 1 to 100 million cells in the bloodstream (4). Although their existence has been known since the nineteenth century, it is only the recent development of modern technologic platforms that has permitted the reliable capture and characterization of CTCs. A major advantage of CTCs compared with other minimally invasive assays is their potential utility as a liquid biopsy serving multiple critical functions (5). Although beyond the scope of this article, important consideration should be given to CTCs, not just as PD endpoints, but also as prognostic markers, predictive and intermediate endpoint biomarkers of response (6).

For example, studies have validated the prognostic significance of CTC counts in several tumor types, including breast, colorectal, and prostate cancers, providing further evidence of their tumoral origins (7–9). The U.S. Food and Drug Administration (FDA) approval of baseline CTC counts, as assessed with CellSearch technology as a prognostic marker in metastatic breast, colorectal, and prostate cancers, heralded the emergence of CTC enumeration as an analytically validated tool in clinical practice.

Although their utility as predictive and intermediate endpoint (or surrogate) biomarkers remains under investigation, they represent areas of great promise and potential. For example, Maheswaran and colleagues were able to detect the emergence of the acquired EGF kinase domain T790M drug-resistance mutation in CTCs in the majority of patients with metastatic non–small cell lung cancer (NSCLC) who had clinical tumor progression while receiving tyrosine kinase inhibitor treatment, suggesting that the molecular analysis of CTCs offers the possibility of monitoring changes in tumor genotypes during treatment (10). Such potential to detect secondary genetic aberrations that may lead to drug resistance with CTCs suggests a possible role as putative predictive biomarkers.

In addition, during the COU-AA-301 phase III clinical trial of the CYP17 inhibitor abiraterone (Zytiga; Janssen Biotech) in post-docetaxel–treated patients with castration-resistant prostate cancer (CRPC), CTCs were enumerated at several time points (baseline, weeks 4, 8, and 12) during treatment. CTC conversion (≥5 to ≤1 CTCs per 7.5 mL of blood) was associated with improved survival as early as 4 weeks after treatment commencement. An analysis of a biomarker panel involving CTCs and lactate dehydrogenase (LDH) levels undertaken at 12 weeks demonstrated survival surrogacy by meeting all Prentice criteria (criteria defined by Prentice that a surrogate marker must satisfy to be useful in clinical trials; ref. 11) at the individual patient level (12). If confirmed in other phase III clinical trials, these CTCs in combination with LDH may be the very first robust surrogate biomarker for survival in this disease setting, which would be of major significance in the rapidly changing therapeutic landscape of CRPC.

Validation and Technical Aspects of CTCs

Validation and Technical Aspects of CTCs

Validation of CTC assays

Validation of a biomarker involves the acquisition of evidence that within a predefined context of use, the biomarker is integral in a specific aspect of drug development. Such a validation process, with the final objective of determining the clinical relevance of the biomarker, is critical in its development. According to the National Cancer Institute (NCI) Biomarker Task Force, biomarkers are qualified as "valid" if the analytic system has well-established performance characteristics and there is consensus in the scientific community on the significance of results; "probably valid" if the evidence only seems to elucidate the significance of results; and "exploratory" in all the rest of cases (3). Most assays exploring CTCs are unfortunately still exploratory and lack analytic and/or clinical validation.

Recently, a panel of international experts summarized the recommendations for the development and validation of CTCs as biomarkers (13). The first requisite in biomarker development is the presence of an analytically validated assay. Pre-analytic (collection of specimens, storage, and handling), analytic (sensitivity, specificity, or reproducibility), and post-analytic (data analysis and reference intervals) variables should be assessed, ideally in multicenter
collaborative studies. An analytically validated assay should then undergo clinical validation in the setting of clinical trials, aiming to link the information the biomarker provides to specific biologic or clinical outcomes. For example, clinical studies evaluating CTC protein expression as PD biomarkers will need to establish the baseline variability associated with the assay through the use of technical and biologic duplicates, to determine if the PD effects observed are indeed due to the drug being assessed, or if CTC heterogeneity is a potential issue. Furthermore, it will be critical for any new CTC technologies, which are not approved by the FDA, to undertake robust healthy volunteer studies to assess the false-positive CTC detection rates.

Isolation and enrichment of CTCs

Several methods have been developed for the evaluation, isolation, and enrichment of CTCs in blood, based on the physical and chemical properties of these cells. Because CTCs are extremely rare cells in the bloodstream, enrichment techniques have been used for separation from peripheral blood cells. Identification of CTCs is then performed, through either immunofluorescence, reverse transcription PCR (RT-PCR), or other techniques involving sophisticated software and microscopy. Enrichment and isolation technologies that are validated or which are currently in development are summarized in Table 1.

Affinity binding approaches use antibodies that either bind to the surface of cells expressing specific antigens (positive selection by capturing EpCAM or CK 9, CK19-positive cells, or negative selection by specifically eliminating cells that express the leukocytic antigen CD45), or attach to the magnetic beads for separation based on magnetic fields (immunomagnetic assays; Fig. 1). Microfluidic platforms (CTC-chips) are based on devices with antibody-coated microstructures, which allow the mixing of blood cells through the generation of microvortices to significantly enhance the number of interactions between target CTCs and the antibody-coated chip surface (14–16). Such an approach enables the capture of large numbers of viable CTCs in a single step from whole blood without the need for an initial enrichment step.

Other CTC capture platforms are based on other physical properties such as size (using microfilters that isolate CTCs based on their greater size), density, or decreased deformability of CTCs compared with erythrocytes and leukocytes (Fig. 1; ref. 17). Another development has been the use of nanodetectors bound with EpCAM antibodies, which are inserted into a peripheral vein, thus increasing the volume of blood that is in contact with the detector and thereby allowing the capture of greater numbers of CTCs (18). A different approach uses dielectrophoretic methods, based on the assumption that CTCs have different electric properties and can therefore be separated from normal cells by applying electric fields (19). Recently, novel assays that target a combination of physical (size) and biologic (immunomagnetic) properties of CTCs have been developed, such as the CTC-iChip, which is capable of sorting rare CTCs from whole blood at a rate of 10 million cells per second in both epithelial and nonepithelial cancers (20). The European consortium CTCTrap has also developed a platform involving a functionalized antibody-containing three-dimensional (3D) matrix that combines immunocapture and size-based separation for CTC enumeration and characterization, including CTC culture (21).

Identification of CTCs

Most CTC assays use an immunofluorescence-based method that defines CTCs as nucleated cells [positive for the nuclear dye 4',6-diamidino-2-phenylindole (DAPI)] that are positive for epithelial markers [cytokeratins (CK) and EpCAM] and negative for the leukocyte markers (CD45). These need to be undertaken by trained operators selecting CTCs based on fluorescent microscopy, although automated digital microscopy systems are able to detect CTCs in a relatively reliable and efficient fashion (22, 23). The HD-CTC platform (Epic Sciences, Inc.) is a novel platform that does not rely on any single protein enrichment strategy. Instead, all nucleated cells are retained and immunofluorescently stained with anti-CK, anti-CD45, and anti-DAPI antibodies and imaged in a high definition scanner. This enables multiple parameters to be analyzed for the characterization of specific populations of CTCs (Fig. 1; ref. 24).

Functional assays are also currently available, based on the detection of secreted proteins by CTCs, and can potentially specifically detect viable cells and discard apoptotic ones (EPISPOT; ref. 25). Other approaches for CTC identification are based on targeting specific mRNA with RT-PCR (26). This strategy requires semi-quantitative assays, as nonmortal cells are able to express the targeted transcripts, albeit at a reduced level. The detection of tumor-specific DNA aberrations in CTCs has also been explored and could potentially be the most specific approach, although concerns exist about intra- and interpatient tumor heterogeneity (27).

The CellSearch System

The only FDA-cleared assay to date is the CellSearch System, an immunomagnetic system that has been developed for the quantification of CTCs in whole blood samples (Fig. 1). The CellSearch system defines a CTC according to its size, positivity for EpCAM and CK, and negativity of CD45 expression. CTC enrichment is performed using immunomagnetic antibodies against EpCAM. CTC identification is performed by a trained operator with a fluorescence microscope, after immunofluorescent labeling with antibodies against CD8, 18, 19, and 45, and DAPI. Results are expressed as the number of CTCs per 7.5 mL of whole blood (28).

High reproducibility of the assay, as shown by an inter-assay variation coefficient (CV) of 12% and an interinstrument CV of <20%, has been reported. The CV between laboratories ranges however from 45% to 64%, mainly due to discrepancies in sample reporter analyses with high numbers of apoptotic cells (29). An algorithm for automated CTC enumeration has been developed, with 0% reported variability of the automated algorithm compared...
Table 1. CTC assays classified by their respective underlying mechanisms

<table>
<thead>
<tr>
<th>Assay</th>
<th>Developer</th>
<th>Comments (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Immunomagnetic assays: positive selection—EpCAM antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CellSearch&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Janssen Diagnostics</td>
<td>EpCAM-coated beads based positive selection using magnetic beads followed by staining and image analysis (92)</td>
</tr>
<tr>
<td>AdnaTest</td>
<td>AdnaGen</td>
<td>Immunomagnetic enrichment (EpCAM, MUC-1, Mesothelin) and subsequent molecular profiling (93)</td>
</tr>
<tr>
<td>Anti-EpCAM/anti-CK antibody CTC enrichment</td>
<td>Glenn Deng, Stanford University</td>
<td>CTC enrichment assay using the combination of anti-CK and anti-EpCAM antibodies (13)</td>
</tr>
<tr>
<td>MACS</td>
<td>Miltenyi Biotec</td>
<td>Bound by antibodies against a ligand of asialoglycoprotein receptor, and subsequently magnetically labeled by magnetic beads (84)</td>
</tr>
<tr>
<td>Dynabeads Epithelial Enrich</td>
<td>Life Technologies</td>
<td>Uses the monoclonal antibody BerEP4 against the EpCAM antigen (95)</td>
</tr>
<tr>
<td>CellCollector</td>
<td>Gilupi</td>
<td>Functionalized structured medical wire coated with anti-EpCAM antibodies placed directly into the blood stream of a patient via an indwelling catheter, remains in the arm vein for 30 minutes and thus enables the capture of CTCs &lt;i&gt;in vivo&lt;/i&gt; (96)</td>
</tr>
<tr>
<td>Biofluidica CTC Detection System</td>
<td>Biofluidica</td>
<td>EpCAM-coated chip to capture followed by release of cells and electrical counting (97)</td>
</tr>
<tr>
<td><strong>2. Immunomagnetic assays: negative selection—CD45 antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPISPOT Lab</td>
<td>Laboratoire de Virologie, Hôpital Lapeyronie, CHU Montpellier</td>
<td>Detects proteins secreted/released/shed from viable cells after the depletion of CD45&lt;sup&gt;+&lt;/sup&gt; and selecting based on the expression of EpCAM (98)</td>
</tr>
<tr>
<td>Aviva CTC Enrichment Kit</td>
<td>AVIVA Biosciences</td>
<td>Combination of size-based RBC depletion and WBC depletion (99)</td>
</tr>
<tr>
<td>Precelleon</td>
<td>Precelleon</td>
<td>Red cell lysis step followed by immunomagnetic labeling, and subsequent depletion of CD45&lt;sup&gt;+&lt;/sup&gt; cells (100)</td>
</tr>
<tr>
<td>Dynabeads CD45 Negative Enrichment OMS Rare</td>
<td>Life Technologies, Cleveland Clinic, Sterncell Technologies</td>
<td>Paramagnetic beads covalently coupled to anti-human CD45 antibody that enable isolation or depletion of CD45&lt;sup&gt;+&lt;/sup&gt; leucocytes (95)</td>
</tr>
<tr>
<td>Negative RARE (RosetteSep-Applied Imaging Rare Event)</td>
<td></td>
<td>Red cell lysis, immunomagnetic labeling, and subsequent depletion of CD45&lt;sup&gt;+&lt;/sup&gt; cells (13)</td>
</tr>
<tr>
<td><strong>3. Microfluidic chips</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OncoCEE</td>
<td>Biocept</td>
<td>Biotin-tagged antibodies that bind selectively to CTCs (102)</td>
</tr>
<tr>
<td>ClearCell FX System</td>
<td>Clearbridge</td>
<td>Label-free technology that uses lateral traps to capture tumor cells based on size and deformability (103)</td>
</tr>
<tr>
<td>ClearID</td>
<td>Cynvenio</td>
<td>High-throughput microfluidic sheath flow isolation technology by ferrofluid with cell staining plus downstream DNA analysis via NGS or qPCR (104)</td>
</tr>
<tr>
<td>Isoflux CTCChip</td>
<td>Fluxion Biosystems</td>
<td>Microfluidic chip based on immunomagnetic capture (105, 106)</td>
</tr>
<tr>
<td>Herringbone-Chip</td>
<td>Daniel Haber and Mehmet Toner, Dana-Farber and MGH</td>
<td>Microvortices are used to significantly increase the number of interactions between target CTCs and the antibody-coated chip surface (15)</td>
</tr>
<tr>
<td><strong>4. Size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ScreenCell</td>
<td>ScreenCell</td>
<td>Microporous membrane filter allows size selective isolation of CTCs (107)</td>
</tr>
<tr>
<td>CellSieve</td>
<td>Creatyl Microtech</td>
<td>Lithographically fabricated filters with precision pore dimensions (108)</td>
</tr>
<tr>
<td>CellOptics ISET</td>
<td>Ikonyx RareCells</td>
<td>Automated imaging platform combined with size-based isolation (109)</td>
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| **(Continued on the following page)**
with a median 14% variability with the manual process, and has been used to evaluate the significance of morphologic changes in CTCs (30, 31).

The Utility of CTCs as PD Biomarkers

In general, CTCs have been used as PD biomarkers through two main strategies, either indirectly via CTC enumeration or directly through the molecular characterization of CTCs.

Enumeration of CTCs for PD studies

Although an indirect marker of drug-associated effects, changes in CTC levels before and during treatment have been used to reflect active doses of a novel antitumor agent in different tumor types, including breast, colorectal, and prostate cancers. In CRPC, enumeration of CTCs has been used as an indirect PD biomarker for both androgen receptor (AR)- and non-AR–targeting drugs. For example, CTC enumeration before and during treatment was incorporated in single-agent abiraterone and enzalutamide phase I/II clinical trials to demonstrate active doses of drug. These CTC declines not only confirmed PD effects of these drugs, but were also an early indicator of antitumor activity, as demonstrated by their correlation with significant prostate-specific antigen (PSA) declines (32–34). CTCs are especially useful in such a setting as patients with advanced CRPC generally have bone-predominant disease and therefore lack objectively measurable soft tissue disease (34).

CTC enumeration was also used as a PD and potential intermediate endpoint biomarker in a recent phase II trial of the novel MET/VEGFR2 inhibitor cabozantinib, where an adaptive design was used to determine the lowest active daily dose of drug to administer to patients with metastatic CRPC. In this study, although PSA responses by PCWG2 criteria only occurred in 1 of 34 (3%) patients with advanced CRPC, 58% of patients who received a lower dose of 40 mg of cabozantinib had a CTC conversion from unfavorable to favorable categories, providing confirmation of the PD effects of cabozantinib at that dose, which was also associated with clinical benefit, supporting its use as an intermediate endpoint biomarker (35).

Because individual patients will have different CTC counts, future studies should also consider using the relative changes of CTC counts to monitor PD effects (e.g., >30% reduction to indicate treatment response), rather than just looking at changes from unfavorable (≥5) or favorable (<5) CTCs per 7.5 mL of blood (36).

Table 1. CTC assays classified by their respective underlying mechanisms (Cont’d)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Developer</th>
<th>Comments (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parsortix</td>
<td>Angle</td>
<td>5. Size and deformability: Uses size and deformability using a weir-type step filter (111)</td>
</tr>
<tr>
<td>ClearCell FX</td>
<td>Clearbridge</td>
<td>Label-free technology that uses lateral traps to capture cells based on size and deformability (112)</td>
</tr>
<tr>
<td>OncoQuick</td>
<td>Greiner Bio</td>
<td>Porous barrier density gradient centrifugation technology (113)</td>
</tr>
<tr>
<td>Maintrac SIMFO</td>
<td>One</td>
<td>Erythrocyte lysis, isolation by centrifugation, and incubation with a fluorescence-labeled antibody against EpCAM (114)</td>
</tr>
<tr>
<td>MagSweeper</td>
<td>Stanford University</td>
<td>Immunomagnetic enrichment of target cells. Individual extraction of isolated cells based on their physical characteristics (115)</td>
</tr>
<tr>
<td>ApoStream</td>
<td>ApoCell</td>
<td>Micro-channel flow fields are used to isolate CTCs through dielectrophoresis field flow fractionation (DEP-FFF; ref. 19)</td>
</tr>
<tr>
<td>DEPArray</td>
<td>Silicon Biosystems</td>
<td>Arrayed electrodes generate spherical DEP cages. CTCs are detected and sorted. Detection and sorting of rare cells and sorted by morphologic parameters (116)</td>
</tr>
<tr>
<td>CellSelect</td>
<td>CellTrafic</td>
<td>Uses telomerase-specific replication-selective adenovirus for detection of CTCs (117)</td>
</tr>
<tr>
<td>TelomeScan</td>
<td>Sysmex</td>
<td>Detection of CTC by using a virus that emits fluorescence on replication (118)</td>
</tr>
<tr>
<td>PACS Synergyx</td>
<td></td>
<td>Polymer-antibody cell separation. Combines patented cell-specific antibodies with a polymer coat (119)</td>
</tr>
<tr>
<td>HD-CTC</td>
<td>Epic Sciences, Inc</td>
<td>No single protein enrichment strategy. All nucleated cells are stained by immunofluorescence and imaged in a high-definition scanner (24)</td>
</tr>
</tbody>
</table>

Abbreviations: MGH, Massachusetts General Hospital; RBC, red blood cells; WBC, white blood cells.
Molecular characterization of CTCs for PD studies

PD studies of antitumor molecular therapeutics have involved the characterization of drug effects on cell membrane antigens on CTCs and/or the selective reduction of genetically distinct subpopulations of CTCs. The molecular characterization of CTCs has involved a range of different techniques, including the assessment of protein expression by immunofluorescence or immunohistochemistry (6, 37). There are a range of protein-based assays, including HER2 (38), γ-H2AX (39), EGFR (40), and insulin-like growth factor-1 receptor (IGF-IR; 41) expression, as well as AR signaling (42) on CTCs, which have been incorporated as exploratory PD biomarkers in clinical trials and are highlighted here in this section.

HER2 expression. HER2 expression on CTCs has been extensively tested in patients with breast cancer in different disease settings. During the GeparQuattro clinical trial, CTC HER2 expression was evaluated in patients with HER2-positive early breast cancer before and after neoadjuvant chemotherapy/trastuzumab treatment using the CellSearch system (43). Initial validation studies were undertaken with breast cancer cell lines with known HER2 gene amplification status. CTCs were considered to overexpress HER2 if at least one CTC showed strong (3+) HER2 immunofluorescence. Fourteen of 58 (24%) patients were found to overexpress HER2 on CTCs, including 8 (14%) patients with HER2-negative primary tumors and 3 (5%) patients after trastuzumab treatment. Interestingly, CTCs that were scored HER2-negative or weakly HER2-positive before or after treatment were present in 11 of 21 patients with HER2-positive primary tumors.

In view of the potential for HER2 status to change during disease recurrence or progression in patients with breast cancer, reevaluation of HER2 expression on CTCs may be an important strategy. Different CTC assays have been evaluated, with varying results. For example, the CellSearch and AdnaTest BreastCancer assays were prospectively evaluated for HER2 expression in 221 patients with metastatic breast cancer (44). Overall, only 62 (28%) of 221 patients were CTC-positive in both assays and, of these, 13 (21%) had HER2-positive CTCs with both platforms. Concordance in HER2 status between both assays was observed in only 31 (50%) of these 62 CTC-positive patients (P = 0.96). The authors attributed this lack of correlation in CTC HER2 assessment to several reasons, including technical differences between both assays. For example, while the CellSearch assay evaluates the HER2 status of individual CTCs by...
immunofluorescence, the AdnaTest BreastCancer assay determines the average HER2 expression of all tumor cells, and is therefore unable to detect heterogeneity in HER2 expression between different CTCs and to establish the percentage of HER2-positive CTCs (44).

Other studies have indicated that CTC counts seem to be higher in patients with estrogen receptor–positive (ER⁺) breast cancer, in contrast to HER2-positive and triple-negative breast cancers, which could potentially be explained by low EpCAM expression and a more mesenchymal phenotype of tumors belonging to the basal-like molecular subtype of breast cancer, and therefore not detectable by most current methods (45, 46). A novel HER2-based microfluidic device for the isolation of CTCs from peripheral blood of patients with HER2-expressing solid tumors has recently been developed as an alternative to EpCAM-based CTC capture methods (47).

A phase III clinical trial (DETECT III) is ongoing that evaluates CTC counts before first- to third-line treatments in patients with metastatic breast cancer found to be HER2-negative in the primary tumor. Patients with ≥1 HER2-positive CTC before the start of a new line of chemotherapy will be randomized to chemotherapy alone versus chemotherapy plus lapatinib with a primary endpoint of progression-free survival (PFS) superiority (NCT01619111; ref. 48).

γ-H2AX expression and other markers of apoptosis. The expression of the nuclear DNA double-strand break damage biomarker γ-H2AX on CTCs has been assessed in patients with a range of tumors receiving cytotoxic chemotherapies and PARP inhibitors. The evaluation of PD changes was undertaken through CTC enumeration and the assessment of percentage of γ-H2AX–positive CTCs. Out of 11 of 15 patients with CTCs identified, γ-H2AX–positive CTCs were detected in 6 patients (% of γ-H2AX–positive CTCs among CTCs: 1.6%–31%; ref. 49). The PD effects of chemotherapies with or without a PARP inhibitor were assessed over time in 5 patients. There were increased γ-H2AX–positive CTCs found in all patients after treatment [mean of 2% (range, 0%–6%) at baseline; 38% (range, 22%–64%) posttreatment].

Wang and colleagues recently presented data on the utility of CTC-based PD biomarkers in phase I and II studies of targeted therapies conducted by the NCI (50). Only 30% of patients participating in eight NCI phase I and II studies in a variety of solid tumors were statistically evaluable because of insufficient CTCs collected at baseline. Also, when considering specific PD biomarkers from multiple studies involving topoisomerase-1 and PARP inhibitors, the γ-H2AX–positive CTC baseline level was less than 20% in 34 of 50 patients. The fraction of CTCs expressing γ-H2AX—indepedent of changes in the total CTC count—increased in patients receiving treatment with different topoisomerase-1 inhibitors either alone or in combination with other drugs. Interestingly, correlations between γ-H2AX levels and antitumor responses were observed in patients with refractory cancers in a phase II randomized trial of the combination of veliparib and cyclophosphamide (50).

Other markers, such as RAD51, M30, and phosphorylated histone H3, may also potentially be used as functional readouts of apoptosis but are limited by the number of CTCs that have to be analyzed. Such biomarkers may enable the monitoring of the apoptotic-inducing and DNA-
damaging effects of drugs such as PARP and ATM inhibitors over time (37).

**EGFR expression.** EGFR expression on CTCs has been demonstrated in a number of tumor types using the CellSearch® system, including patients with advanced breast, prostate, colorectal, and lung cancers (40, 45, 51, 52). For example, serial sampling of a patient with advanced colorectal cancer treated with the EGFR inhibitor panitumumab had a decrease in the number of EGFR-positive CTCs (40). Nevertheless, heterogeneity and variability in EGFR expression on CTCs have been issues that have hampered its utility as a PD biomarker. In addition, a lack of concordance in EGFR expression has been observed between CTCs and the primary tumor and associated metastases. For example, of 9 patients with EGFR-negative CTCs, 6 had EGFR-positive metastases, while the available primary tumor specimens for 3 patients were also EGFR-positive (53).

Procurement of tissue representative of tumor is a particular issue in lung cancer, where accessibility to the primary cancer is often inaccessible. There is thus great interest in the utility of a number of different sources of surrogate tissues including CTCs and lung lavage specimens. Lung lavage and blood samples were collected from patients with NSCLC and analyzed on the VeriFAST platform (54). Signal intensity of EGFR expression seemed to be less heterogeneous among CTCs than the lavage specimens, although this could reflect differences in tumor burden.

**IGF-IR expression.** We have previously incorporated the evaluation of IGF-IR–positive CTCs as an exploratory end-point in patients treated on a phase I study with the IGF-IR monoclonal antibody figitumumab (CP-751,871; Pfizer; ref. 41). The CellTracks system was adapted to incorporate antibodies to detect IGF-IR immunofluorescence. The assay was initially validated using cell lines with known levels of IGF-IR expression and spiked blood samples from healthy volunteers, before incorporation in three phase I trials of figitumumab administered as a single agent or with chemotherapy involving patients with metastatic cancers. Importantly, the diagnostic antibody was shown not to interfere with figitumumab as they target different IGF-IR epitopes. One of the limitations of this assay was the binary classification of CTCs as either positive or negative for IGF-IR, and thus further work will be required to enable better quantification of IGF-IR immunofluorescence.

**AR signaling.** Miyamoto and colleagues molecularly characterized CTCs that were isolated using microfluidic chip technology and subsequently analyzed by immunofluorescence for PSA and prostate-specific membrane

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**Table:**

<table>
<thead>
<tr>
<th>CellSearch system</th>
<th>ISET filtration device</th>
<th>DTCs</th>
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<tbody>
<tr>
<td>DAPI</td>
<td>CK</td>
<td>CD45</td>
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**Figure 3.** Examples of immunofluorescence images of CTCs and disseminated tumor cells (DTC) from patients with CRPC. Top two panels demonstrate CTCs enriched with the CellSearch CTC Test; third and fourth panels demonstrate CTCs isolated using the ISET filtration device; bottom, DTCs from a bone marrow trephine biopsy specimen. ISET, isolation by size of epithelial tumor cells. Images were obtained using an automated fluorescence microscope scanning system Bioview Duet (Bioview Ltd.). Images courtesy of Mateus Crespo, the Institute of Cancer Research (Sutton, Surrey, United Kingdom). CD45, leukocyte common antigen.
antigen (PSMA). In a retrospective analysis of 12 patients treated with abiraterone, the presence of >10% CTCs with a mixed AR signature (PSA- and PSMA-positive) was associated with a worse overall survival compared with patients with fewer AR-mixed CTCs (42). However, as compelling as these results may seem, they are associated with several limitations, namely the nonvalidated CTC isolation assay, CTC selection based on EpCAM, and the fact that cell viability, which may substantially affect such results, was not taken into account. An alternative approach that may be used is to quantify AR expression directly on CTCs using different platforms, as shown in Figs. 2 and 3. It is possible to identify varying AR localization, to the nucleolus when ligand bound and activated, or the cytoplasm when inactive, even in CTCs from the same patient with CRPC (Fig. 2). Such assessments may be particularly relevant in the assessment of novel targeted agents in CRPC, such as enzalutamide, which reduces the efficiency of AR nuclear translocation.

Considerations When Incorporating CTCs as PD Biomarkers

A framework that may be used for the incorporation of CTCs into early-phase clinical trials is the Pharmacological Audit Trail (PhAT) that we have previously described (1, 55). The PhAT links all key stages of drug development and assesses the risk of failure in a step-wise approach as sequential questions are addressed, permitting key "go, no-go" decisions to be made (Table 2). CTC PD studies could initially be incorporated as exploratory endpoints in early-phase clinical trials. This will provide investigators with invaluable insights into their CTC technologies by applying the assay in the clinic, without affecting medical or drug development processes.

Implementing CTCs in early-phase clinical trials as PD endpoint biomarkers requires that several key elements be fulfilled, namely (6):

- CTCs need to be detectable in the patient population;
- CTCs need to be collected and processed using an analytically validated standardized assay, for example, the CellSearch platform and using a uniform definition of what is called a CTC;
- CTC viability needs to be assessed (56–59) and apoptotic and nonapoptotic CTCs need to be differentiated (Fig. 2);
- PD endpoints can either be based on CTC enumeration, expression of drug target or a surrogate on CTCs, or a combination of both factors (60, 61);
- Ideally, the PD endpoint should be demonstrated in both CTCs and tumor biopsies (60).

Looking to the Future: Promises and Limitations of CTCs

Promises of CTCs as PD biomarkers

The main advantage of using CTCs as PD markers lies in their potential role as the "leukemic phase" of solid tumors (62). We should thus be able to use CTCs to study the PD interactions between a novel antitumor therapeutic and its intended target directly in representative tumor cells without requiring invasive tumor biopsies. In the future, isolating such CTCs through technologic advances in single cell profiling will allow us to go beyond simple cell enumeration and the characterization of protein markers on CTCs to measure treatment effects. For example, recent studies have shown that it is possible to monitor tumor genomes by using array comparative genomic hybridization (CGH) and next-generation sequencing (NGS) technologies (63). As tumor cell genomes are prone to change in response to antitumor treatments, we could implement this technology to measure PD and other effects, which may be especially relevant in the identification of genomic markers associated with treatment resistance (Fig. 4). Other future applications of CTCs may also include the study of PD effects of a treatment ex vivo by generating primary cell cultures from CTCs, also known as CTC-derived xenografts (CDX), which may allow the testing and validation of multiple PD markers using unique patient-derived tumor models.

Limitations of CTCs as PD biomarkers

Despite recent advances in the field of CTC isolation and characterization, it has been suggested that CTC studies may be limited in their role as PD biomarkers as they may not always be meaningful representations of bona fide tumor tissue or more aggressive tumor cells. For example, immunomagnetic assays are highly specific methods but can potentially miss CTCs that do not express the target antigen (6). Such tumor types include melanoma and sarcoma, which do not express epithelial surface antigens; in such cases, CTC enrichment based on alternative cell surface markers, such as CD146 for melanoma, or vimentin for sarcoma have been proposed (64, 65). Similarly, aggressive tumor cells undergoing epithelial–mesenchymal transition (EMT) are known to lose the expression of epithelial markers and would therefore not be detected by an EpCAM antibody-based enrichment technique (66). Therefore, using a CTC isolation method based on EpCAM-positive cell enrichment may cause some bias against such tumor cells (67). Although EpCAM-negative CTCs and novel CTCs assays tailored for specific tumor types may potentially address these issues, such novel platforms still required analytic validation and/or clinical qualification (Table 1).

Other novel CTC subpopulations that also need further characterization with novel platforms in the future include small CTCs (CK+, CD45−, intact DAPI), which are morphologically similar in size to white blood cells (WBC) and could be missed by filters and other size/density capture devices (Fig. 3). Such CTC capture platforms that isolate CTCs based on their size, density, or decreased deformability compared with erythrocytes and leukocytes are potentially fast and economic methods, but have the disadvantage of having an overlap in physical properties with nontumoral cells in the blood (17).
Until such issues are addressed, clinical studies incorporating the use of CTCs should remain focused on current validated platforms, such as the CellSearch platform, which is based on EpCAM-positive cells enrichment. The use of CTCs isolated with such methods to study PD treatment effects nevertheless poses several challenges, which will need to be resolved:

1. Viability of CTCs isolated with current platforms. A variable and significant proportion of CTCs captured with the CellSearch assay has been described as apoptotic, and its proportion has been shown to rise with increasing CTC counts (60, 68). However, it is not clear whether this is a purely in vivo phenomenon or if this is due to the multiple processing steps undertaken during CTC enrichment and isolation with the CellSearch platform. Regardless, a comparison of the latter technology with other isolation methods to formally clarify the role of sample processing in increasing the proportion of apoptotic CTCs needs to be undertaken.

2. CTC heterogeneity. The heterogeneity of epithelial malignancies is now well established and CTCs are likely to represent a subset of cells derived from heterogeneous primary tumor cells that survive in the circulation (69–71). The clonal heterogeneity of CTCs has been confirmed at the genomic level through the observation of a substantial variability in...
chromosomal abnormalities (68, 72). However, in some cases, such genomic abnormalities may actually be homogeneous as in the case of ERG rearrangements in CTCs from advanced prostate cancer, suggesting a potentially common clonal origin of CTCs and metastatic disease in ERG-rearranged prostate cancers (60). Phenotypical pleomorphism in CTCs has also been described, and good examples are certain cell-surface markers such as IGF-IR, EGFR, or HER2 expression, or intracellular markers such as AR or phosphorylated Histone-H3 with positive and negative cells coexisting in the same patient (41, 42, 52, 73, 74).

3. **Biologic differences between CTCs and solid tumor lesions.** In some cases, CTCs may differ in their phenotype between primary and metastatic tumors, such as with HER2-positive CTCs in HER2-negative primary and metastatic breast cancer (75). In addition, there are potential limitations with the interpretation of the actual impact of antitumor therapies on CTCs versus solid tumor lesions. This may be due to the contamination of CTCs with another cell population because of drug-related active mobilization of tumor cells to the blood or the passive shed of cells from the tumor surface. There is also higher exposure of the blood compartment to a systematically

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**Figure 4.** Envisioning the future utility of CTCs in modern oncologic treatment pathways. As CTC methods and technologies for the characterization of single tumor cells continue to improve, we envision the incorporation of CTCs into clinical studies for the identification and monitoring of tumor heterogeneity, clonal evolution, and treatment selection in individual patients. In the future, it may become possible to treat an individual patient rationally with sequential targeted therapy regimens based on the identification of evolving tumor characteristics, as extrapolated from CTCs, which may be isolated and studied at multiple time points during the patient’s treatment journey. As illustrated by Treatment A in the figure, blood samples may be collected at baseline for phenotype and genomic profiling studies to dissect tumor heterogeneity, so as to select the most relevant target(s) across all the clones for initial antitumor treatment. Such studies may include the analyses of the tumor mutational landscape, such as NGS technologies, whole genome copy number variation studies using array CGH with validation by FISH or quantitative PCR (qPCR), and/or gene expression profiling. To select the most appropriate treatments, data from these studies will be interrogated with modern bioinformatic tools and compared with accessible cancer genome databases linked to treatment effects and patient outcomes. So as to guide subsequent treatment decisions, blood samples may be drawn again at both early and late time points to study antitumor effects, as well as the development of early and late mechanisms of resistance in different tumor clones. Furthermore, coclinical studies with CDX models may be developed by engrafting CTCs in immunosuppressed mouse in parallel to the investigations described above. Following engraftment and an initial growth of the CTC graft, tumors may then potentially be expanded to develop sufficient tumor mass for biobanking, comparative molecular characterization of the CDX with the primary tumor, and for the assessment of a range of treatments to identify mechanisms of resistance to guide future treatment regimens. Adapted from Tentler et al. (121) by permission of Macmillan Publishers Ltd. Access the most recent version of this article at: http://www.nature.com/nrclinonc/index.html.
administered drug, in contrast to the solid tumor component.

As discussed, CTCs may be reliably detected in the majority of patients with metastatic prostate (41), breast (7), and colorectal cancers (8) using the FDA-approved CellSearch technology. CTCs have also been detected in patients with other tumor types, such as small cell lung cancer (76). However, only about 10% of patients with NSCLC have of ≥5 CTC/7.5 mL for enumeration, limiting the wider application of CTCs in this patient population (77). Gainor and colleagues (78) discuss such issues relating to lung cancer in their article in this in this CCR Focus section.

In the future, another application of CTC technology may emerge in the field of minimal residual disease diagnostics after patients have completed curative local treatment. For example, in breast cancer, a clinical trial has been initiated to investigate the addition of trastuzumab in patients with detectable CTC counts after adjuvant treatment (NCT01548677). Nevertheless, it remains unclear if residual CTCs have the same phenotype as the primary tumor and if CTCs represent a primary resistant clone, or if these CTCs are truly independent of the targeted pathway (79). Also, at earlier disease stages, CTCs are often absent or only present at very low frequencies using enrichment and detection tools currently available; sensitivity will thus need to be significantly improved and platforms appropriately validated before CTC technology can be applied in such scenarios (80).

Clinical trial designs that incorporate CTCs as PD biomarkers should bear in mind that large numbers of patients may be necessary to draw definitive conclusions from such studies, which may potentially be hampered by cell heterogeneity due to the attrition of patients without CTCs or low viable CTCs counts. Other important limiting factors with the inclusion of CTCs as PD biomarkers are the significant costs involved with CTC kits, operator time and complexities of CTC capture and evaluation. Nevertheless, in the future, it is likely that as CTC platforms become more sensitive and economical, CTC evaluation will increasingly become an integral part of translational clinical studies and patient management.

Other Biomarkers

In addition to CTCs, other blood components are also excellent sources of information about the cancer and host that may be used as potential biomarkers, including PD endpoints. For example, platelet-rich plasma has been incorporated successfully in early-phase clinical trials for PD studies involving selective signaling inhibitors (81). Other examples include the isolation of circulating nucleic acids such as circulating tumor DNA (ctDNA), as discussed by Figg and Newell in this CCR Focus edition (82), which is increased in patients with advanced cancers compare with healthy individuals (83), micro-RNA or the characterization of gene expression changes that nonmalignant blood cells undergo in response to micro- and macro-environmental changes induced by the tumor (84, 85). Recently, two gene expression signatures with prognostic utility, linked to the inflammatory and immune response, were developed and validated in CRPC (86, 87). Depending on future testing, these signatures may even potentially hold some utility as PD and predictive biomarkers for immunotherapy treatments. Apart from blood-borne biomarkers, van der Veldt and Lammersma (88) discuss the use of in vivo imaging of taxanes as PD biomarkers in this of CCR Focus section. In addition, Hertz and McLeod (89) also discuss the use of pharmacogene panels to detect germline SNP biomarkers, while Low and colleagues (90) detail genome-wide association studies (GWAS) as a tool to identify common genetic variants associated with drug toxicity and efficacy in cancer pharmacogenomics in this CCR Focus section.

Conclusions

It is envisioned that the future use of CTCs as PD biomarkers will not simply be confined to enumeration, but also include their routine molecular characterization in early-phase clinical trials. Overall, the assessment of CTC-based PD biomarkers has potential for rapidly demonstrating proof-of-mechanism during the clinical development of molecularly targeted anticancer therapeutics in “real-time.” However, clinical trials using CTCs as PD endpoints clearly demonstrate that the interpretation of data across multiple studies using different CTC isolation and molecular characterization technologies comes with numerous challenges (37, 91). Prospective studies with uniform and standardized definitions of CTCs are thus urgently needed. Such studies should exploit the full potential of CTCs not just as PD biomarkers, but also as prognostic, predictive, and intermediate endpoint markers.

Authors’ Contributions

Conception and design: T.A. Yap, D. Lorente, A. Omlin, D. Olmos, J.S. de Bono

Development of methodology: T.A. Yap, D. Lorente, J.S. de Bono

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.A. Yap, D. Lorente, J.S. de Bono

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Writing, review, and/or revision of the manuscript: T.A. Yap, D. Lorente, A. Omlin, D. Olmos, J.S. de Bono

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.A. Yap, D. Lorente

Study supervision: T.A. Yap, D. Lorente

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## Circulating Tumor Cells: A Multifunctional Biomarker

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