Circulating Tumor Cells as Biomarkers in Prostate Cancer

Daniel C. Danila¹,³, Martin Fleisher², and Howard I. Scher¹,³

Abstract

Unmet needs in prostate cancer drug development and patient management are the ability to monitor treatment effects and to identify therapeutic targets in a tumor at the time treatment is being considered. This review focuses on establishing analytically valid biomarkers for specific contexts of use in patients with castration-resistant prostate cancer (CRPC), emphasizing a biomarker currently in clinical use, circulating tumor cells (CTC). The FDA Critical Path provides a road map for these investigations, which, if followed, will facilitate the incorporation of these types of assays into clinical decision-making. CTC enumeration at baseline and post-treatment is prognostic of survival, with no threshold effect, and the shedding of cells into the circulation represents an intrinsic property of the tumor, distinct from extent of disease. 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 antiandrogen 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. Additionally, we discuss novel technologies to enrich and characterize CTCs from more patients, the potential clinical uses of CTCs in determining prognosis and monitoring treatment effects, and CTCs as a source of tissue to identify predictive markers of drug sensitivity to guide treatment selection. Prospective studies, designed around the biomarker itself and the specific clinical context for which it is applied, are needed to further assess the role of these and novel markers in clinical practice. Clin Cancer Res; 17(12); 3903–12. ©2011 AACR.

Introduction

Prostate cancer has distinctive difficulties in drug development and patient management; this is because the standard imaging modalities used to assess disease in bone, the most common site of spread, have not been standardized, and because the directional change in prostate-specific antigen (PSA) levels, the most frequently altered biomarker in the disease, may not reflect the status of disease accurately (1, 2). Up to 20% of men with castration-resistant prostate cancer (CRPC), who eventually respond to a systemic cytotoxic therapy proven to prolong life, have an initial PSA increase before the decline (3, 4); the decline may not occur for up to 12 weeks (5) or not occur at all, with immunomodulatory agents proven to prolong life and that are postulated to slow disease trajectory in part through effects on the tumor microenvironment (e.g., sipuleucel-T; ref. 6). It is, therefore, not surprising that the association between a given post-therapy change in PSA and survival is modest and, that it is not, appropriately, accepted by regulatory agencies for drug approvals (7, 8). Finding more reliable indicators of disease status is a critical unmet need.

Although it has been particularly encouraging to see several drugs with diverse mechanisms show a survival benefit in phase III trials (9), it is also clear that these agents are active in subsets of patients, and that distinct cohorts remain that are resistant to therapy de novo. This situation, coupled with the recognition that the molecular determinants of a tumor that contribute to growth may change as the disease progresses, presents the additional unmet need to profile an individual patient’s tumor at the time treatment is considered (10). It is our hypothesis that specific blood-based circulating tumor cell (CTC) assays, which are obtained in the context of routine clinical practice, can fulfill these unmet needs.

Before the role of any assay in medical decision-making can be determined, it is essential that the assay itself meet specific and rigorous performance requirements. This review is focused on the process of establishing the analytic validity of CTC biomarkers for specific contexts of use in patients with CRPC, looking first at what is currently available, and next at other assays that are at different
points in the development process. For a discussion of bone turnover, urinary, and tissue-based biomarkers, the reader is referred to key reviews (11–16).

A disease framework

Prostate cancer is a heterogeneous disease that can span decades in some or be lethal in a relatively short time in others. The clinical states model provides a framework to reduce heterogeneity and define therapeutic objectives for an individual patient or group of patients and to guide drug development (17, 18). This model is the basis of the Prostate Cancer Working Group 2 (PCWG2) guidelines (Fig. 1; ref. 8). Importantly, the framework incorporates established standards of care and enables clinical trial questions to focus on the unmet needs for specific patient groups at different points in the disease continuum. The PCWG2 guidelines also recommend reporting outcomes on the basis of changes in each disease manifestation (e.g., PSA, soft tissue, and bone) individually. That recommendation enables each parameter to be studied alone or in combination with other measures in relation to a clinical outcome.

Contexts of use for biomarkers. The road map for biomarker development is outlined in the Oncology Biomarker Qualification Initiative (OBQI), a collaboration between the U.S. Food and Drug Administration (FDA), Centers for Medicare & Medicaid Services (CMS), and National Cancer Institute (NCI), described in the FDA Critical Path Initiative (19). It requires a longitudinal process that starts with developing a robust assay, which is analytically validated, and separately, prospective trials developed for the specific biomarker question(s) for the context of use in which the biomarker is being evaluated. A draft guidance was recently issued by the FDA (20). The specific contexts of use in which qualified biomarkers would influence medical decisions include the following:

1. Detection, use of the biomarker to establish a diagnosis;
2. Prognosis, measuring the probability of a specific clinical outcome, such as recurrence, progression, or survival;
3. Prediction, identifying the chance of response to a specific therapy;
4. Response-indicator biomarkers show a pharmacologic or physiologic response from the treatment (e.g., a decline in PSA), which does not necessarily mean that the patient has benefitted from a treatment;
5. Efficacy-response biomarkers are surrogates of how a patient feels or functions or how long he survives, extrapolating the clinical benefit;
6. Treatment resistance biomarkers define biologic determinants of failure or progression, such as second site mutations.

Analytic validation establishes the performance characteristics of an assay and the range of conditions under which an assay gives reproducible and accurate data. In this process, it requires rigorous performance testing to meet
Clinical Laboratory Improvement Amendments (CLIA) regulatory requirements. The validation process requires rigorous performance that can be considered in three steps: (1) preanalytic assessment of specimen selection, handling, processing, and storage parameters; (2) validation of analytic characteristics to meet CLIA regulatory requirements, establishing the inter- and intrasay precision, linearity, analyte recovery, and standardization, and developing a comprehensive quality control program; (3) postanalytic parameters require data management and storage.

Clinical qualification, distinct from the analytic validation, is the evidentiary process of linking a biomarker with biological processes or clinical endpoints, in the context of an intended use, to inform a medical decision as described in the FDA Critical Path (19, 21).

As there are more biomarkers than questions, and given the effort and cost to conduct these investigations, it is essential to develop metrics to determine which biomarkers that seem promising warrant prospective testing in large-scale phase III trials to establish qualification.

**Enumerating circulating tumor cells.** CTCs are rare events in the peripheral blood of patients with a variety of metastatic carcinomas and are currently estimated to account for 1 cell in a billion nucleated cells. In contrast to invasive procedures like biopsies, a blood test for CTCs is safe and can be done frequently (Fig. 2).

Numerous CTC isolation and capture techniques have been reported, but only one method, CellSearch (Veridex LLC), is analytically valid (22) and cleared by the FDA for use. The assay enriches CTCs on the basis of antibodies to epithelial cell adhesion molecule (EpCAM) conjugated to magnetic beads, which are further classified as CTCs on the basis of morphologic limits and rigorous criteria for staining for cytokeratin (CK-6, 8, 18), of displaying a nucleus on magnetic beads, which are further classified as CTCs on the epithelial cell adhesion molecule (EpCAM) conjugated to antibodies to safe and can be done frequently (Fig. 2).

In a separate cohort of patients treated at Memorial Sloan-Kettering Cancer Center (MSKCC), CTC number considered as a continuous variable was also shown to be an independent prognostic factor for survival with no threshold effect (Fig. 3; ref. 34). This argues against the use of discrete CTC cutoff values in decision making, a finding confirmed in a reanalysis of patients included in the IMM38 prostate cancer study (trial registration NCT00133900), upon which the prostate cancer clearance was based (33). For this analysis, baseline pretreatment variables associated with high risk of death were high lactate dehydrogenase (LDH) concentration [hazard ratio (HR) 6.44, 95% confidence interval (CI) 4.24–9.79], high CTC count (HR 1.58, 95% CI 1.41–1.77), high PSA levels (HR 1.26, 95% CI, 1.10–1.45), low albumin (HR 0.10, 95% CI, 0.03–0.39), and low hemoglobin (HR 0.72, 95% CI, 0.64–0.81). In the post-treatment analysis at 4 weeks, 8 weeks, and 12 weeks, the changes in CTC number from baseline were strongly associated with risk, whereas changes in PSA were weakly or not associated. The most prognostic factors for survival were baseline LDH levels and the fold change in CTC count from baseline to the follow-up time of measurement (concordance probability estimate, 0.72–0.75; ref. 33). The finding was consistent at baseline, 4, 8, and 12 weeks post-therapy, which suggests that CTC number measured at 4 or 8 weeks following treatment provides important prognostic information and can potentially be used as an indicator of a favorable or unfavorable outcome with therapy. In these analyses, the
CTC biomarker (baseline or fold change from baseline) was more prognostic than PSA, significantly improving on prior models used to predict the overall survival (OS) probability among patients with metastatic CRPC (35).

Data from phase II investigations, although often encouraging, do not establish surrogacy. Now needed are large-scale phase III trials in which a survival benefit is shown and with the CTC biomarker question embedded. Such a stepwise approach was studied in the context of the clinical development in CRPC of abiraterone acetate, a17α-hydroxilase and 17,20-lyase inhibitor, which inhibits androgen biosynthesis in the testis, adrenal gland, and

Figure 2. CTCs in patients with prostate cancer. Sampled by phlebotomy at the time when treatment is being considered, CTCs have the potential to provide tumor material for molecular profiling for biomarkers informative of tumor sensitivity to the targeted therapy being considered. FISH images are reproduced from Leversha et al. (53).
prostate tumor (9). On the basis of activity in a phase I study (trial registration NCT00473512; ref. 36), the CTC enumeration biomarker was incorporated in 2 independent phase II trials at MSKCC (trial registration NCT00485303) and the Royal Marsden Hospital (trial registration NCT00474383). Patients with CRPC progressing after docetaxel-based chemotherapy were treated with abiraterone acetate with baseline CTC frequencies of ≥5 cells/7.5 mL of 70% (95% CI, 54–81) and 79% (95% CI, 63–90) of patients, and a similar post-therapy conversion rate from unfavorable to favorable counts of 34% (95% CI, 20–53) and 41% (95% CI, 25–59) in the 2 trials, respectively (37, 38). Similar post-therapy CTC conversion rates of 49% (95% CI, 35–63) were found in a phase I and II trial (trial registration NCT00510718) of patients with CRPC treated with MDV3100, a novel AR antagonist specifically developed by investigators at MSKCC for activity in prostate cancer cells with overexpressed AR (39). These findings speak to the analytic validity of the assay and formed the basis for exploring CTC enumeration as a potential efficacy–response (surrogate) biomarker of survival for AR-targeted therapies in patients with CRPC progressing post-docetaxel treatment.

Through a longstanding relationship with the FDA, CTC enumeration has entered the first formal collaborative effort to prospectively establish it as an efficacy-response biomarker. As discussed with the Biomarker Qualification Review Team ("A Voluntary Data Submission to Support the Qualification of Circulating Tumor Cells (CTCs) as an Efficacy-response Biomarker in Castration Resistant Prostate Cancer (CRPC)," December 2009), the statistical analysis plan toward qualification of CTC number as an efficacy-response surrogate for OS requires the biomarker to fully capture the net effect of treatment on OS (40), in multiple prospective trials (41). The data analysis of the first randomized phase III trial of patients with postchemotherapy-treated CRPC randomized to receive abiraterone acetate versus placebo (trial registration NCT00638690), which incorporated a CTC biomarker question, was discussed in a face-to-face meeting at Center for Drug Evaluation and Research (CDER). The trial showed prolonged survival (HR 0.65, 95% CI, 0.54–0.77), time to PSA progression (HR 0.58, 95% CI, 0.46–0.73), and radiographic progression-free survival (HR 0.67, 95% CI, 0.58–0.78; ref. 42). The data from this first trial will be used to generate the biomarker panel that best explains the survival outcome. A second randomized phase III trial treating patients with progressive CRPC postdocetaxel with MDV3100 (trial registration NCT00974311), and including an embedded CTC biomarker as an efficacy-response question, has completed accrual. Once the survival analysis for the primary efficacy question has been completed, the relationship between the biomarker panel identified in the first trial (Cougar AA-301) will be studied (43). Ultimately, multiple trials of similar design will be required to establish surrogacy.

The successful completion of this qualification process will show the clinical utility of monitoring CTC changes
with treatment, as an intermediate endpoint for detecting survival benefit from AR-targeted therapies. To extend the context of use, additional surrogacy analyses are embedded in phase III randomized survival-based trials, which have the CTC biomarker question embedded, such as TAK-700 (trial registration NCT01193257), and immunotherapy-based therapies such as ipilimumab (trial registration NCT01057810). In addition to an efficacy-response biomarker qualification effort, changes in CTCs have been proposed as a pharmacodynamic readout of immune-based therapies, such as the recently FDA-approved vaccine therapy with sipuleucel-T for patients with CRPC (44). **Molecular profiling of circulating tumor cells.** In addition to providing prognostic information and a potential indicator of efficacy, CTCs have the potential to provide a snapshot of the molecular makeup of an individual patient's tumor, to profile for determinants that predict for sensitivity or resistance to treatment. The molecular determinants that contribute to tumor growth can change during the course of disease. Consequently, to effectively deliver the appropriate targeted approach for an individual patient, it is essential to profile the tumor at the time of treatment decision. Doiing so is currently limited by the lack of reliable assays for the biomarkers being studied, and the difficulty in obtaining representative tumor samples in a routine clinical practice setting (45). CTCs isolated from the peripheral blood or disseminated tumor cells from the bone marrow (46, 47) of patients with prostate cancer at any stage are of particular interest, because they have the potential to provide tumor material representative of a molecular snapshot of the disease.

Though early work suggested that TMPRSS2-ERG rearrangements are sufficient to initiate prostate neoplasia (24, 48), as are linked ETS rearrangements for more aggressive disease (48), the reported clinical significance of TMPRSS2-ETS rearrangements has been inconsistent. In preclinical animal models, aberrant expression of ERG alone was insufficient to initiate transformation by itself (49), but it was sufficient when concomitant with phosphoinositide 3-kinase activation or PTEN loss (50). Additionally, copy number increase of chromosome 21, with and without rearrangement for TMPRSS2, was associated with high Gleason grade and advanced stage, reflecting generalized aneuploidy (49, 51). Unlike KRAS mutations predicting primary resistance to epidermal growth factor receptor (EGFR) target therapy, no known markers have been shown to predict for resistance to a specific therapy in CRPC. AR amplification, although infrequent in primary and/or diagnostic tumor specimens, is detected in upwards of 50% of castration-resistant lesions (10, 52). AR genomic amplification and copy number gain have been documented in CTCs from patients with CRPC, with frequencies similar to those reported for late-stage tumors (24, 53). Our initial exploratory analysis suggested that AR amplification and copy gain, occurring under the selective pressure of androgen deprivation therapy, might predict for sensitivity to a second-generation AR antagonist (54). Additionally, cells that have initiated true amplification events also seem to develop the increased chromosomal instability, as shown by additional MYC, TMPRSS-ETV, and PTEN abnormalities (24, 53). Similarly, copy number alterations have been strongly associated with more aggressive tumors, as described in the ongoing comprehensive analysis in the Prostate Oncogenomics Project at MSKCC (55). The prognostic value of HER2/neu, EGFR, and insulin like growth factor (IGF)-1R expression in CTCs is currently being studied in the context of clinical trials with novel therapies targeting these determinants as a potential tumor-sensitivity predictive biomarker, as well as a pharmacodynamic measurement for defining the optimal dose beyond the maximum tolerated dose (23, 53, 56, 57). Although promising, full qualification will ultimately require analytical validation of the assays used, as well as new prospective trials to explore the relationship between the specific biomarker measured and clinical outcome. **Moving beyond CellSearch.** Following the FDA Critical Path, each new assay needs to be analytically validated and clinically qualified in the intended context for which the biomarker will be used. Most studies with CellSearch in prostate cancer have evaluated patients with CRPC. CRPC includes patients who have progressed on one or more hormonal therapies, those who are about to start a cytotoxic drug, and those who have progressed in multiple therapies (Fig. 1). Table 1 shows the frequency of favorable and unfavorable counts for these patient groups, highlighting a need to develop assays that detect cells in more patients and at a higher frequency (32–34). On the basis of this need, other technologies are being developed to increase CTC detection rates and to obtain more highly enriched samples, such as filtration (38, 58), microfluidics (59–61), and fluorescence-activated cell sorting (FACS; ref. 62; Table 2). Through optimization of the flow in the capturing device, recent CTC chips have improved detection of CTCs in early stages of prostate cancer, including in the context of localized disease (59, 60).

The results obtained are typically compared with CellSearch as the standard technology, and for CTC enumeration. Notable is that most of the "new" approaches detect "CTC" in patients with "favorable" (0-4 cells/7.5 mL of blood) counts using Cell Search. Not described clearly is the fact that the "CTC" definitions in different assays are not the same and that they may not be capturing the same cells. As such, each "new" assay will be required to undergo its own analytical validation before it can be considered for clearance or qualification in any disease context (Fig. 2). Little appreciated, however, is that different CTC assay technologies are not measuring exactly the same biomarker, and, thus, each new assay is, in essence, proposing a new definition of a CTC, as there is currently no "standard."

Our group developed and validated a FACS-based method that isolates and captures EpCAM⁺, CD45⁻ cells from a mononucleated layer separated through Ficoll-Hypaque gradient from blood collected from patients with CRPC. DAPI is used as a vital stain to exclude permeable and apoptotic cells. The cells isolated by FACS were shown by multiplex reverse transcriptase (RT)-PCR to express
prostate-specific mRNAs (PSA, AR, TMPRSS2), indicating that these EpCAM\(^+\) events are \textit{bona fide} CTCs. In parallel samples collected from 124 patients with metastatic CRPC, this FACS-based method isolated an average 100-fold more putative CTCs than did CellSearch, and more CTCs could be isolated in a larger cohort of patients: \(>50\) events per sample in 58% versus 10% of patients, or \(>10\) events per sample in 88% versus 32%, by FACS versus CellSearch.

### Table 1. Flow cytometric circulating tumor cell enrichment captures more events

<table>
<thead>
<tr>
<th>FACS event range</th>
<th>Total ((N = 124))</th>
<th>Chemotherapy exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre ((n = 33))</td>
</tr>
<tr>
<td>0–4</td>
<td>8 (6%)</td>
<td>6 (18%)</td>
</tr>
<tr>
<td>(\geq 5–9)</td>
<td>7 (6%)</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>(\geq 10)</td>
<td>109 (88%)</td>
<td>25 (75%)</td>
</tr>
<tr>
<td>(&gt;50)</td>
<td>72 (58%)</td>
<td>11 (33%)</td>
</tr>
</tbody>
</table>

**CellSearch Events “Meets CTC Definition”**

| 0–4      | 74 (60\%) | 27 (82\%) | 13 (49\%) | 34 (54\%) |
| \(\geq 5–9\) | 10 (8\%) | 3 (9\%)    | 2 (8\%)   | 5 (7\%)   |
| \(\geq 10\) | 40 (32\%) | 3 (9\%)    | 12 (45\%) | 25 (39\%) |
| \(>50\) | 13 (10\%) | 1 (3\%)    | 7 (28\%)  | 5 (8\%)   |

**NOTE:** In a cohort of 124 patients with CRPC, FACS sorting and analysis of EpCAM\(^+\), CD45\(^-\), and DAPI\(^-\) events increased the detection rate and the absolute number of events compared with CellSearch enrichment.

### Table 2. Selected circulating tumor cell enrichment and characterization techniques

<table>
<thead>
<tr>
<th>Method</th>
<th>Technology</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic separation based on</td>
<td>CellSearch system</td>
<td>CTCs are characterized on the basis of rigorous morphologic criteria, based on staining for cytokeratin (CK-6, 8, 18), nucleus (DAPI), and excluding white blood cell (CD45 staining). The only FDA-cleared CTC technology as an aid in monitoring of patients with metastatic breast (26–29), colorectal (30, 31), and prostate cancer (32, 33).</td>
</tr>
<tr>
<td>on EpCAM-Ab-coupled ferrofluid</td>
<td>MagSweeper</td>
<td>Exploratory enrichment method based on ferro-magnetic conjugated antibodies (63).</td>
</tr>
<tr>
<td>Antibody-coupled microposts</td>
<td>EpCAM-based separation</td>
<td>Microposts and herring-bone CTC chips allow sensitive and selective detection of CTCs for enumeration and further molecular profiling at genomic, transcriptional, and translational levels (59, 60, 64).</td>
</tr>
</tbody>
</table>
| PSMA-based separation         |                              | Isolation of CTCs based on PSMA expression allows enrichment of EpCAM-negative tumor cells (61). Staggered obstacles optimize cell-size dependent flow to maximize enrichment of CTCs.
| Negative selection            | RARE detection method        | Depletes erythrocytes and CD45-positive white blood cells,agnostically negatively enriching tumor cells (65).                                                                                                                                                     |
| RT-PCR                        | Prostate-specific gene       | PSA mRNA detection in peripheral blood of patients with prostate cancer (66–68).                                                                                                                                                                                          |
| expression profiling           | Micropore filtration         | Isolation based on microfiltration separates CTCs on the basis of size (38, 58, 69)                                                                                                                                                                                       |
| Imaging-based methods         | EPISPOT assay                | Immunospot-based method of detecting CTCs on the basis of secretion of proteins (PSA from prostate CTCs; ref. 70).                                                                                                                                                         |
|                               | FACS                         | Multimarker cell analysis and isolation for further molecular profiling (62, 71, 72).                                                                                                                                                                                       |
|                               | Scan microscopy              | High-speed scanning for detection of CTCs by fluorescence microscopy (73, 74).                                                                                                                                                                                            |

**Abbreviations:** PSMA, prostate-specific membrane antigen; RARE, RosetteSep-Applied Imaging Rare Event.
respectively (Table 1). Of particular interest is that these approaches have the potential to enable in-depth molecular profiling in CTCs in chemotherapy-naive patients, in which isolating CTCs with higher purity will facilitate the study of gene signature patterns prognostic or predictive of sensitivity to a targeted drug, or define the mechanism of acquired resistance that occurs under the selective pressure of a targeted therapy. Additionally, these sensitive technologies may allow clinical testing for the risk of relapse with distant metastasis on the basis of shedding of CTCs at the time of primary treatment of the prostate cancer, and guide the decision on adjuvant therapy on the basis of post-treatment CTC enumeration.

**Future directions.** The approval of new therapies for patients with prostate cancer based on OS requires large cohorts of patients with lengthy follow-up. Qualified biomarkers for discrete contexts of use have the potential to shorten the drug development process. To do so requires validated assays and, separately, the design and conduct of prospective clinical trials that generate evidence toward the qualification. More significant power to discriminate between low and high risk of a specific outcome could be obtained by combining multiple biomarkers, such as LDH, albumin, and PSA with baseline CTCs (33, 34), which will ultimately result in a “basket” biomarker meant to influence medical decision-making.

Because it is not clear which biomarker will be most informative for a medical decision to address a specific context of use, there must be a process for prioritizing biomarker development. A biomarker should be tested in phase I and II efficacy trials to measure the robustness of its association with predicted clinical outcome in the selected context of use, before beginning full development of the biomarker in large, costly phase III trials.

**Disclosure of Potential Conflicts of Interest**

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