Molecular Characterization and Clinical Utility of Circulating Tumor Cells in the Treatment of Prostate Cancer

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OVERVIEW

Circulating tumor cells (CTCs) are rare cancer cells that can be detected in the blood of patients with solid malignancies. The Veridex CellSearch Assay was analytically and clinically validated, and has received U.S. Food and Drug Administration (FDA) clearance for the enumeration of CTCs in breast, colorectal, and prostate cancer. A number of alternative assays, with potential advantages, are currently undergoing clinical and/or analytic validation before their routine use can be established. In prostate cancer, high pretreatment CTC counts have been associated with worse survival, and changes in CTC counts in response to treatment have been established as indicators of response to treatment. Additional analyses are ongoing to establish the value of CTC counts as a surrogate of survival in prospective, phase III trials, which could influence the process of drug development and regulatory approval. Additionally, CTCs have a potential role in the molecular characterization of prostate cancer, serving as "liquid biopsies" to determine the molecular characteristics of the disease. The study of androgen receptor (AR) mutations or amplification, chromosomal rearrangements, or the determination of DNA repair biomarkers has been evaluated in clinical trials. CTCs have a wide range of potential applications, from their prognostic use in stratification of patients in clinical trials or the assessment of response to treatment, to the pharmacodynamic evaluation of novel agents, or the discovery and use of predictive biomarkers that can aid in the development of personalized medicine.

Prostate cancer currently represents an exciting area of clinical research, with substantial improvements in our understanding of the molecular biology of the disease that have led to the approval of several new agents in recent years. However, these improvements have stressed the importance of developing adequate biomarkers for patient selection and the assessment of response.

Current recommendations for the assessment of outcome and the design of endpoints in clinical trials were developed by the Prostate Cancer Working Group (PCWG) and are summarized in the PCWG2 Criteria. These are based on a composite endpoint that takes into account imaging (CT scans and bone scans), prostate-specific antigen (PSA) levels, and clinical outcomes. These criteria have important limitations, especially in the large proportion of patients with metastatic castration-resistant prostate cancer (CRPC) that present with exclusively bone metastatic disease not measurable by RECIST criteria. PSA has been shown to correlate weakly with survival, is not adequate to guide treatment in the first 12 weeks, and could be less reliable in more advanced, potentially less AR-driven stages of the disease. Confirmatory bone scans are required at least 6 weeks after the appearance of new lesions to exclude a “flare reaction” in response to treatment.

In addition to these limitations in the evaluation of response, the high prevalence of bone-exclusive disease has traditionally hindered our ability to obtain tissue for molecular analysis. Current histologic analyses are usually performed in the original diagnostic biopsies, and have not taken into account clonal selection and acquired resistance mechanisms. Bone marrow biopsies, although feasible in the setting of specialized research units, are not routinely used in daily practice.

CTCs are extremely rare cells present in the blood in an estimated frequency of one in a few million blood cells, originating from shedding from the original tumor. An FDA-cleared assay, the Veridex CellSearch System, was approved based on studies performed in breast, colorectal, and prostate cancers and is available for the enumeration of CTCs.

The use of CTCs as prognostic and treatment-response biomarkers has been proposed in the CRPC setting; their potential use as a surrogate of survival could also accelerate the development of active agents. Their use as a "liquid biopsy" for molecular characterization may assist in the development of new agents.
opment of precision medicine by assessing the molecular biology of the disease in real-time and personalizing treatment on a patient-to-patient basis.

METHODS FOR ISOLATION AND QUANTIFICATION OF CTC

Assays for quantitative analysis of CTC from blood samples typically require an initial preparatory step (centrifugation, washing, and addition of cellular preservatives to delay CTC entering apoptosis for up to 72 to 96 hours), a second enrichment or isolation step (which can be based on immunoaffinity or on the particular physical properties of CTCs), and last a semi-automated image-based approach for identification.

The most common approach for isolation of CTC is based on immune-magnetic systems; samples are incubated with specific antibodies to select certain cell populations and are afterwards separated by magnetic means. This may be performed through positive selection (by conjugation with antibodies against epithelial cell adhesion molecules [EpCAM], expressed in most CTC and not in other blood cells) or negative selection (using antibodies against leukocyte-expressed antigens such as CD45). Selection based on antibody binding to specific prostate cancer proteins, such as prostate-specific membrane antigen (PSMA), can also be utilized.

Alternatively, technologies have been developed to isolate CTC from blood samples based on their different size, deformability capacities, or electrical properties of CTC compared with other blood cells, mainly leukocytes. The principal limitation of these approaches is a lower discriminatory capacity as a result of some overlap in physical properties, and the dependence of the results on the blood flow rate, which results in lower sensibility and specificity. Although, these techniques allow for isolation at a lower cost when compared with antibody-based approaches.

Recently, enrichment-free systems for study of CTCs have emerged. Fiberoptic scanning enables high-throughput assaying of the entire population of cells in blood without requiring any protein-based selection but only erythrocyte lysis, being therefore less vulnerable to loss of cells. One of the reasons for developing these approaches is the direct study of clusters of circulating cells rather than individual CTCs.4

Evolution of isolation techniques has led to the development of systems for composite selection, where cells are first targeted by conjugation with EpCAM antibodies with selection being based on their size through a multistep obstacle architectural design.5

The CellSearch System (Janssen Diagnostics, LLC, Raritan, NJ)6 is however, to date, the only FDA-cleared technology for quantification of CTCs, with demonstrated clinical relevance in breast, prostate, and colorectal cancer, and has become the standard comparator for any novel platform in development. The CellSearch System is based on an automated immunomagnetic enrichment and staining system: anti-EpCAM and anti-creatinine kinase (CK) antibodies are used for positive selection, complemented by counterstaining with anti-CD45 antibodies to discard leukocytes.

A current challenge for CTC enumeration is to address the effect of tumor heterogeneity in CTC. Systems based on positive selection for epithelial markers may be missing those CTCs that have undergone epithelial-mesenchyme transition (ETM), which arguably representatives of the more aggressive clones of disease and also have limited applicability in nonepithelial malignancies (e.g., sarcomas or melanoma). Platforms using several concomitant antibodies for a composite selection (including markers that are not repressed during EMT) would help in addressing this pitfall, although this would affect the monetary cost of the assay.

The final step of any quantitative analysis also requires an image-based system with the input from a human operator to identify CTCs among the selected cells. Algorithms for completely automated counting of CTCs are being optimized.6

Widely accepted characteristics to define a CTC include: round to oval morphology, size greater than 5 μm, a visible nucleus (4#,6-diamidino-2-phenylindole positive), positive staining for cytokeratins 8, 18, and/or 19 (phycoerythrin), and negative staining for CD45 (allophycocyanin). Modifications of this definition may increase the numbers of isolated cells, but may weaken the prognostic value of the enumeration.7

To assess the validity of the CellSearch System, Kraan et al analyzed aliquots of the same six blood samples drawn from patients with metastatic cancer in 14 different laboratories. Interestingly, inconsistency in scoring was mainly derived from the manual interpretation by a trained operator of the events identified by the semiautomatic system, especially in those samples containing a high number of dead or apoptotic cells.8

Overall, among the different approaches for isolation,
there is probably not a single ideal method, but selection of the appropriate method would depend on the intended downstream application.

**CTC COUNTS AS PROGNOSTIC AND TREATMENT-RESPONSE BIOMARKERS**

The value of CTCs as a prognostic and predictive biomarker has been validated in studies across multiple cancer types. As a result of potential variability in the determination of CTCs, threshold values have generally been proposed to distinguish “favorable” from “unfavorable” counts.

The IMMC38 study was the first to evaluate the prognostic and predictive role of CTC enumeration using the CellSearch System in CRPC patients. In the study, 164 patients starting a new cytotoxic chemotherapy regimen were eligible and CTC counts were determined in 3- to 4-weekly intervals. An unfavorable pretreatment count (≥ 5 CTC in 7.5 mL of blood) predicted a worse overall survival (OS) than a favorable count (< 5 CTC in 7.5 mL of blood) after adjusting for known prognostic values (Eastern Cooperative Oncology Group [ECOG] status, hemoglobin, lactate dehydrogenase [LDH], and alkaline phosphatase) in multivariate analysis. The predictive value of a CTC conversion from an unfavorable to a favorable count at different time points was also explored. Patients that maintained a favorable count at all draws had the longest OS (26 months), followed by those that converted from unfavorable to favorable (21.3 months), those that converted from favorable to unfavorable (9.3 months), and those that maintained an unfavorable count (6.8 months). CTC count was superior to PSA declines in predicting survival, especially in earlier time points. At 12 weeks, receiver operating characteristic (ROC) curve analysis showed a statistically significant superiority of CTC counts over 30% PSA declines in predicting death at 12 months (area under the curve [AUC] 81.5 vs 67.3%; p = 0.022). A second analysis of the same study evaluated the prognostic value of baseline CTCs as a continuous variable, before treatment initiation and at different time points. After incorporating CTC counts in the multivariate model, only CTC counts and LDH retained clinical significance, which was lost for all other variables, including PSA.10

Alternative cut-off points have also been proposed in evaluating the prognostic and predictive role of CTCs. In a cohort of 99 metastatic CRPC patients treated at the Memorial Sloan-Kettering Cancer Center (MSKCC), there was a strong correlation between baseline CTC number and survival, without a threshold effect. Baseline CTC counts were modestly correlated with other indicators of disease burden, such as baseline PSA values and bone scan index.11 Another single-center study performed in the Royal Marsden evaluated 119 CRPC patients undergoing CTC enumeration, and reported a statistically significant difference in OS in patients with a CTC count less than 5/7.5 mL, 5 to 50/7.5 mL, and greater than 50/7.5 mL at baseline and after the first and second cycle of treatment. Additionally, a decline of 30% in CTC was also associated with improved survival in patients with a baseline unfavorable CTC count. Baseline CTC counts were associated with other known baseline prognostic factors, such as high alkaline phosphatase levels, low hemoglobin, high PSA, prior cytotoxic chemotherapy, or the presence of bone metastases.12

The prognostic and predictive value of CTCs using the 5 CTC/7.5 mL cutoff proposed in the IMMC38 study was prospectively validated in the phase III COU- AA-301 trial, evaluating abiraterone against placebo in the postchemotherapy setting. CTC conversion was associated with improved OS as early as 4 weeks after commencing treatment. The inclusion of CTC count changes in the multivariate model significantly reduced the treatment effect at all post-treatment time points, modifying the hazard ratio (HR) for OS in the abiraterone versus placebo groups from 0.74 in the model without CTC count changes to 0.94 in the model that included CTC count changes.13

**CHANGES IN CTC COUNTS AS SURROGATE OF SURVIVAL IN CRPC**

Based on the results of the COU-301 study, a model for assessing CTC response has been developed and evaluated for surrogacy. The degree on which a response biomarker captures the effect of treatment on survival, and can therefore be used in regulatory submissions for novel agents, has been tested using the Prentice Criteria. These criteria require that the biomarker is evaluated in therapies that provide survival benefit, that the treatment has an effect on the proposed biomarker, that the biomarker has an effect on the clinical endpoint, and that the full effect of treatment on the endpoint is captured by the biomarker.14 To qualify as an accepted surrogate for regulatory drug approval, these criteria must be met in a number of large prospective trials, and a meta-analytic approach must prove surrogacy at the trial level as well as at individual level.

A model including CTCs (≥ 5 CTC vs. < 5 CTC in 7.5 mL of blood) and LDH (normal vs. abnormal) at the 12-week landmark time point fulfilled all Prentice Criteria at the individual level. However, proof of surrogacy at trial level requires that these results be reproduced in several large trials.15 If a number of ongoing clinical trials confirm the results of the COU-301 analysis, we may be able to validate the role of CTCs as a response-indicator biomarker, potentially with drug approval based on CTC endpoints. This would increase the efficiency and reduce the costs of the development of novel active agents, and eliminate the bias in OS that would be introduced by treatment in post-trial settings.

**MOLECULAR CHARACTERIZATION OF CTC**

Qualitative assessment of CTC at genomic and proteomic levels provides an insight into biologic processes of the disease and has applications in diagnostic, staging, biomarker discovery, and individualization of treatment. There is great interest in obtaining molecular information from CTCs, as they may constitute a read-out for the cancer molecular un-
derpinnings without requiring the invasiveness of tumor biopsies, and permit longitudinal analyses by collecting sequential samples over time to assess the effect of treatments in tumor evolution. In the field of prostate cancer, molecular characterization of CTCs in parallel to new drug development should bring advances in the current lack of biomarker-driven individualization of treatment.

A wide variety of genome- and protein-based assays can be performed on CTCs, including immunohistochemistry, immunofluorescence, gene copy number analysis via comparative genomic hybridization (aCGH), genome sequencing analysis, and epigenetic studies.

Immunophenotyping of CTCs is the basis of the most implemented assays for enumeration, but also can be used toward their molecular characterization. Multiplexed characterization by immunophenotyping is partially limited by the overlap with antibodies necessary to identify CTCs.

Cytogenetic studies based on fluorescence in situ hybridization (FISH) allow the study in CTCs of the presence or loss (heterozygous or homozygous) of phosphatase and tensin homolog (PTEN), to assess the number of copies of the AR gene, or the presence of erythroblast transformation-specific–related gene (ERG)-based translocations. Multicolored fluorescence permits simultaneous study of these genes, offering a comprehensive profiling of prostate cancer cells, with prognostic value and associated to response to abiraterone acetate in retrospective series.16

Assessment of the AR gene by FISH is complemented by detection of mutations in DNA or altered gene copy number from CTCs.17 Detection of hotspot mutations by targeting sequencing in phosphoinositide 3-kinase (PI3K)/AKT genes in this circulating genomic material would complement the assessment of PTEN function in scrutinizing this highly relevant signaling pathway.18 On of the key interests in assessment of CTCs is the opportunity for both longitudinal assessment (assessing tumor evolution over time as results to treatment-induced selective pressure) and studying intratumor clonal heterogeneity. As an example, detection of emerging mutations in epidermal growth factor receptor (EGFR) in CTCs has served for the study of resistance mechanisms to EGFR inhibitors.

With the rapid technical development of next generation sequencing techniques from circulating nucleic acids19 and the ability to perform single cell whole genome amplifications,20 it is envisioned that CTCs could serve as an easy and economic source for whole genome and transcriptome anal-

**FIG 1. Nuclear γH2AX staining in CTCs as a PD biomarker in the phase I trial evaluating the PARP inhibitor niraparib.**

(A1) Pretreatment and maximal post treatment increase in proportion of CTCs staining positive for nuclear γH2AX in patients with castration-resistant prostate cancer (Part B) with baseline CTC counts of > 3 cells/7.5 mL of blood. (A2) CTCs at baseline from a patient on study with no nuclear staining for γH2AX and the panel. (A3) CTCs from the same patient during treatment with positive nuclear staining for γH2AX. (B1,2) Fresh tumor tissue collected at baseline (B1) and in cycle 2 (B2) stained for γH2AX immunofluorescence (red), showing an increase in the level of γH2AX induction in the post treatment tumor biopsy.

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ysis for diagnostic, predictive, and monitoring for treatment purposes. Lastly, DNA extracted from CTCs would also serve as a source for epigenetic studies, including methylation analysis.21

IMPLEMENTATION OF CTC ANALYSIS IN DRUG DEVELOPMENT

Phase I trials of novel targeted compounds demands biomarker-driven patient selection and markers of antitumor effect with early read-out to optimize drug development programs. The easy access to CTCs offer unique platform for pharmacodynamics (PDs) studies, overcoming the restricted anatomic accessibility to soft tissue or visceral metastases for fresh biopsies in patients with prostate cancer and the limited success rate of bone marrow biopsies in obtaining tumor tissue.

PD assessments in circulating biomarkers allows for monitoring the effect of a drug in tumor cells repeatedly and at different dose levels to determine pharmacokinetic/PD correlations.

Sequential CTC counts to detect early CTC decreases as a surrogate marker of antitumor activity were incorporated in the early phase trials of abiraterone acetate in prostate cancer22 and are now commonly implemented as a PD read-out in many first-in-human trials of drugs in development for prostate cancer. In the case of early trials of figitumumab (CP751,781), a monoclonal antibody targeting insulin-like growth factor 1 receptor (IGF-IR), a mixed quantitative/qualitative approach was attempted, by selectively monitoring CTCs expressing IGF-IR.23

One of the most successful examples of implementation of CTCs for PD studies in early clinical trials is the monitoring of induction of gamma H2A histone family member X (γH2AX) foci in CTC after exposure to DNA targeting agents (Fig. 1) and has contributed to development of several trials of poly ADP ribose polymerase (PARP) inhibitors including patients with prostate cancer.24

CONCLUSION

CTCs have emerged as an important biomarker in current drug development in prostate cancer. Applications in the research and clinical settings are multifold: (1) its prognostic value will be important in the stratification of patients in clinical trials, (2) its value as a surrogate of survival could accelerate drug approval, (3) the rational understanding of the molecular biology can aid in our understanding of prostate cancer and the development of novel agents, and (4) its use as PD biomarkers in early drug development can aid in the selection of biologically active treatment regimens. The application of CTCs in the assessment of response as a tool in clinical decision-making, mirroring its development in breast cancer, has also been proposed. The potential of CTCs for identifying nonresponders earlier than the currently established response biomarkers (CT scans, bone scans or PSA) could be useful in avoiding the administration of toxic and often costly therapeutic options, and receiving subsequent therapy in a more favorable condition, with a better general condition that could increase the likelihood of achieving benefit. See Fig. 2 for a summary of this approach.

Several challenges in the development of CTCs lie ahead. The currently approved CellSearch System, based on the EpCAM positivity of CTCs, could be missing EpCAM negative malignant cells undergoing epithelial-mesenchymal transition, which are potentially relevant in metastatic dissemination. The dependency on a human operator for the counting of CTCs in the CellSearch system has been pointed out as a potential for bias. Novel automated methods in the enrichment and characterization of CTCs may help overcome these issues; however, thorough analytic and clinical validation will be required before their routine clinical use is cleared.

In conclusion, CTCs are among the most promising biomarkers in development in prostate cancer; they are easily accessible and provide material for the assessment of prognosis, response to treatment, and molecular characterization. Further research

FIG 2. Clinical decision making based on CTC results.
will improve the detection and enrichment of CTCs, and may establish their role as surrogates of survival.

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References


