

Clinical utility of circulating tumor cell counting through CellSearch[®]: the dilemma of a concept suspended in Limbo

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Abstract: To date, 10 years after the first demonstration of circulating tumor cells (CTCs), prognostic significance in metastatic breast cancer using the US Food and Drug Administration–cleared system CellSearch[®], the potential utility of CTCs in early clinical development of drugs, their role as a surrogate marker of response to therapy, and their molecular analysis for patient stratification for targeted therapies are still major unsolved questions. Great expectations are pinned on the ongoing interventional trials aimed to demonstrate that CTCs might be of value for guiding treatment of patients and predicting cancer progression. To fill the gap between theory and practice with regard to the clinical utility of CTCs, a bridge is needed, taking into account innovative design for clinical trials, a revised definition of traditional CTCs, next-generation CTC technology, the potential clinical application of CTC analysis in non-validated settings of disease, and finally, expanding the number of patients enrolled in the studies. In this regard, the results of the first European pooled analysis definitely validated the independent prognostic value of CTC counting in metastatic breast cancer patients.

Keywords: CTC, clinical trials, prognosis

Introduction

If one could translate the “Divina Commedia” into a scientific language and try to imagine where Dante Alighieri would have placed circulating tumor cells (CTCs), the answer would be, without a doubt, in limbo. In fact, despite the increasing scientific evidence collected in the last decade, which is enough to avert the danger of Hell, CTC validation into clinical practice is still “far from the light that suits to Heaven”. To date, 10 years after the first demonstration of CTC prognostic significance in metastatic breast cancer using the US Food and Drug Administration (FDA)-cleared system CellSearch[®] (Janssen Diagnostics, LLC, Raritan, NJ, USA),¹ the potential utility of CTCs in the early clinical development of drugs, their role as a surrogate marker of response to therapy and, their molecular analysis for patient stratification to targeted therapies are still major unsolved questions. Furthermore, the very recent disappointing results obtained in the Phase III SWOG S0500 trial, concluding that CTCs are not a good marker in helping to decide when to switch between chemotherapies in women with metastatic breast cancer, now opens a new, intriguing debate.

Insight into SWOG S0500 trial failure

Great expectations are pinned on the ongoing interventional trials aimed to demonstrate that CTCs might be of value for guiding treatment of patients and predicting cancer

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progression.² The results of the SWOG S0500 trial were recently presented at the 2013 San Antonio Breast Cancer Symposium.³ This randomized Phase III trial was designed to test whether persistently high CTC levels after the first cycle of therapy could be used as an early indicator of disease progression and to determine whether switching at that early point to an alternate chemotherapy regimen would result in improved survival and time to progression among patients with metastatic breast cancer. The study confirmed the significant prognostic value of CTCs in the setting of metastatic breast cancer but failed to demonstrate the clinical utility of counting CTCs to evaluate the effectiveness of frontline chemotherapy in metastatic breast cancer patients. Consistently with previously published data, in this trial, patients could be separated into three groups with significantly different prognosis according to levels of CTCs at baseline and after the first cycle of therapy. The study revealed that patients with low baseline levels of CTC had a favorable prognosis, with an overall survival (OS) of 35 months, while an elevated baseline CTC count was associated with a poorer prognosis. Particularly, patients with continued elevated CTCs after one cycle of chemotherapy had the worst prognosis, with a median OS of 13 months, and patients with elevated CTCs at baseline that dropped below the threshold after the first cycle of therapy had an intermediate OS of 23 months.

To test the initial hypothesis, patients who maintained elevated CTC levels 21 days after initiating therapy were randomized either to continue their chemotherapy or to switch to a different, potentially more effective, regimen chosen by the physician. Prior hormonal therapy, bisphosphonate therapy, and targeted therapies for metastatic disease were allowed and were unchanged regardless of CTC levels. One could argue that the design of the study, as previously summarized, might have jeopardized the benefit that switching chemotherapy based on CTC levels might have provided. Furthermore, as the classification of breast cancer has evolved from a single disease to well established molecular subgroups, stratification of patients and therapy selection according to breast cancer subtypes would have been advisable. The idea that the number of CTCs, serially assessed under treatment, might reflect the effectiveness of chemotherapy and predict disease progression early before clinical and radiological evidence is undoubtedly appealing. Although previous studies have demonstrated that CTC assay is indicative of disease progression earlier than conventional imaging, the ability of CTC values at the time point of 3 weeks after starting therapy to assess treatment response has never been explored before. The effect of the type of

anticancer therapy on the prognostic and predictive value of CTCs has not been directly evaluated in the published studies assessing the clinical utility of CTC counting. Particularly, data are not conclusive on the ability of targeted therapies to modify the predictive value of CTC count during therapy. The heterogeneity of first-line treatments allowed in the SWOG S0500 trial might thus have variably influenced CTC behavior. Fluctuations in CTC levels over the course of different therapies prompt caution in interpreting the changes in CTC number soon after (3 weeks) the start of therapy as a biological marker for response to treatment.

To fill the gap between theory and practice with regards to the clinical utility of CTC, a bridge is needed, taking into account a number of strategies, as hereunder reported.

Promising starting points from ongoing clinical trials

The assessment of CTC changes as early markers for resistance to treatment is currently being investigated in the CirCe01 trial,² where patients with high CTC count before the start of the third line of chemotherapy will be randomized between the CTC-driven arm and the standard arm. In the latter, CTC count will be performed after each first cycle, and patients whose CTC count will not drop under the cutoff value will be taken off the therapy and eventually given a further line of treatment, which will be again evaluated by CTC changes after the first cycle of therapy. The trial is based on the assumption that CTC count might detect earlier chemoresistance and promote early chemotherapy changes. To demonstrate the clinical utility of CTCs in driving therapeutic decisions for metastatic breast cancer patients, one could anticipate that results from the CirCe01 trial should be moved from a very palliative to a frontline setting. From a theoretical point of view, it is reasonable that in the first-line setting, the discontinuation of a targeted therapy inhibiting an oncogenic pathway may enhance biological activity in a significant fraction of malignant cells.

This suggests caution in considering repeated early discontinuation of therapy as a strategy to improve patient outcome, supporting the idea that this approach might offer a modest improvement in the treatment of resistant breast cancer.

Insights into tumor biology and, particularly, into the mechanisms underlying sensitivity and resistance to anticancer treatments, come from the molecular characterization of CTCs. The availability of a readily accessible source of biological material might allow the monitoring of the dynamic clonal evolution of cancer and to guide the selection of therapy towards the dominant target. The difference between CTCs and their

corresponding primary tumor with respect to human epidermal growth factor receptor 2 (HER2) status has been previously reported.⁴ In particular, the occurrence of HER2-positive CTCs in patients with HER2-negative primary breast cancer might be of potential clinical value. The DETECT III trial is the first study where treatment choice is made according to the phenotypic characterization of CTCs.³ This ongoing trial has been designed with the aim to evaluate the clinical efficacy of lapatinib, a dual inhibitor of ErbB1 (EGFR [epidermal growth factor receptor]) and ErbB2 (HER2) receptor tyrosine kinases, in HER2-negative metastatic breast cancer patients with at least one HER2-positive CTC. To enroll the predefined sample of patients, more than 1,000 patients will be tested for the presence of HER2-positive CTCs. This will prevent the disappointing results obtained from a previously published Phase II similar trial that reported inconclusive efficacy results due to the limited number of eligible patients.⁵ A critical analysis of the study design might raise the question whether the selected cutoff of at least one HER2 positive CTC is appropriate in the metastatic setting in order to accurately identify a subgroup of HER2-negative breast cancer patients who may derive benefit from anti-HER2 therapies.

A further approach to demonstrate the clinical relevance of CTCs to guide the management of metastatic breast cancer patients is to combine the prognostic information derived from the counting of CTCs with their molecular characterization for tumor markers that predict endocrine sensitivity and resistance. A multi-parameter assay, the CTC-Endocrine Therapy Index (CTC-ETI), has been recently developed that may identify patients with estrogen receptor (ER)-positive metastatic breast cancer who are unlikely to benefit from endocrine therapy.⁶ Using the fourth empty filter in the CellSearch[®] system, ER, BCL-2, HER-2, and Ki-67 as markers for endocrine responsiveness/resistance can be accurately determined and combined with the number of CTCs to calculate the CTC-ETI.

A multicenter trial has been recently opened in an attempt to demonstrate that CTC-ETI at baseline predicts response to endocrine therapy in metastatic breast cancer patients starting a new endocrine therapy. The idea of generating a therapeutic algorithm that combines the well-established prognostic value of the CTC count and the expression of predictive markers on CTCs for endocrine resistance seems intriguing and might provide, if validated, a new valuable criterion for choosing between endocrine therapy and chemotherapy for ER-positive metastatic breast cancer patients.

CTC characterization may also accelerate the drug development process. The COU-AA-301 trial is the first Phase III

study aimed to prospectively evaluate CTC counting as an outcome measure for regulatory approval of a new therapeutic option for metastatic castration-resistant prostate cancer patients previously treated with docetaxel. The COU-AA-301 Phase III trial demonstrated that abiraterone acetate, an irreversible inhibitor of the CYP17 enzyme for androgen synthesis, significantly prolongs OS in metastatic castration-resistant prostate cancer patients progressing after docetaxel therapy.⁷ CTC conversion from an unfavorable to favorable count (using the standard cutoff definition of ≥ 5 CTCs per 7.5 mL) demonstrated a statistically significant impact on OS, suggesting a key role of CTC serial assessment as predictor of survival outcome.

Expanding the definition of traditional CTC

According to the first CellSearch[®] training book, a CTC is characterized by positivity for epithelial cell adhesion molecule (EpCAM), cytokeratins (CKs), and nuclear dye (DAPI [4',6-diamidino-2-phenylindole]); all objects with delineated nuclear image but speckled CK, as well as objects with cytoplasm area which does not surround the nucleus, are defined as “suspicious objects” and are not counted by the operator as CTCs. To date, the significance of these cells is not clear.

The predictive values of all types of suspicious objects were first evaluated in a report by Coumans et al⁸ in a follow-up study performed on 179 patients with castration-resistant prostate cancer. In that study, to evaluate the relationship between different classes of objects and OS, the authors imported the images into the Linux-based software for the CellTracks[®] Analyzer II (Janssen Diagnostics) and used the automated algorithm to identify and reanalyze all objects EpCAM+, CK+, and/or DAPI+. On the basis of morphological and size criteria, the authors established four classes of objects: intact CTCs, granular CTCs, large and small tumor cell fragments (which required the presence of a nucleus or DNA [deoxyribonucleic acid] staining), and large and small microparticles without DNA. The standard size of CK stain was $4 \times 4 \mu\text{m}^2$ as applied in the CellSearch[®] CTC definition, and consequently, the definitions of small and large suspicious objects were adopted. The authors found that all EpCAM+, CK+, and CD45- objects predicted OS in this cohort of patients.

In 2011, our group performed the CellSearch[®] analysis on blood from 25 patients with metastatic renal cell carcinoma and found CTCs in only 16% of patients.⁹ In most CTC-negative patients, we found images of CD45-/CK speckled nucleated objects, or images of CD45- objects with CK

signal not surrounding the nucleus, corresponding to the “suspicious objects” described in the CellSearch® instruction book. This report was the first to suggest that the low number of CTCs detected through CellSearch® in renal cell carcinoma may be due to the presence of a CTC population with atypical characteristics and a peculiar gene expression profile, mainly due to the presence of a speckled CK signal. It has become clear that, by looking only at EpCAM, tumor cells from other major cancers, like melanoma or pancreatic cancer, as well as cells with stemness characteristics, will escape detection through CellSearch®. Besides EpCAM downregulation, the absence or low expression of CKs should be also taken into account as contributing to CTC underestimation using CellSearch®.

Studies performed in breast cancer patients further suggest that the inability of CellSearch® to detect cells which have undergone epithelial mesenchymal transition may explain the absence of CTCs in a subset of patients with metastatic breast cancer with documented progression of the disease.¹⁰

To overcome these pitfalls, Veridex, LLC (Raritan, NJ, USA) in 2011 started working, in collaboration with Massachusetts General Hospital, on next-generation CTC technology which could enhance specificity and sensitivity and enable more extensive characterization of captured cells.

Next-generation technology to improve the performance of CellSearch®

In 2013, Ozkumur et al¹¹ described an inertial focusing-enhanced microfluidic CTC capture platform, termed “CTC-iChip”, capable of sorting, from large volumes of whole blood, rare CTCs with both epithelial and non-epithelial characteristics. The new technology combines the strengths of microfluidics for rare cell handling while incorporating the benefits of magnetic-based cell sorting.

The new system can be run in either a positive selection or a negative depletion mode. The posCTC-iChip was tested in patients with prostate, breast, colon, pancreatic, and lung cancer, showing a very high sensitivity, particularly critical in patients with a lower CTC burden.

Furthermore, the iChip isolates cells in suspension, enabling their immobilization on a standard glass slide for high-resolution imaging and standard clinical cytopathological examination as well as simultaneous staining for multiple biomarkers.

The negCTC-iChip allowed for isolation of CTCs from a non-epithelial cancer, such as melanoma, and from cancer that has undergone epithelial mesenchymal transition and

lost virtually all detectable EpCAM, such as triple-negative breast cancer, providing a comprehensive and unbiased view of non-hematological cells in the bloodstream of cancer patients. In the direct comparison between the posCTC-iChip and the CellSearch® system, the microfluidic device was significantly more sensitive at low CTC numbers, suggesting that a subpopulation of EpCAM-low cells was missed by the CellSearch® bulk-processing approach. The integrated microfluidic technology platform enables the isolation of CTCs, regardless of tumor surface epitopes, and provides an end product that is compatible with both standardized clinical diagnostics and advanced molecular analyses.

The future integration of such an economical chip into a fully automated device would potentially allow broad dissemination of this technology.

The potentiality of CellSearch® fourth channel

There is an additional channel in the standard Veridex CTC kit which may allow the examination of a fourth molecule of interest. To date, this channel has been extensively used to study the apoptotic status of CTCs; for this purpose, CTC assay was integrated with a monoclonal antibody, anti-M30, to recognize a neoepitope in CK-18 that becomes available at a caspase cleavage event during apoptosis and is not detectable in vital epithelial cells. The M30 neoepitope is generally regarded as a stable biomarker, specific for epithelial cell apoptosis.

Rossi et al¹² analyzed M30 expression in breast, colorectal, and renal cancer patients; furthermore, in a small series of breast cancer patients, the change in the number of M30-negative/positive CTC balance was found to correlate with radiologic findings of disease status (progressive versus stable disease/partial response).

Recently, Smerage et al¹³ reported the integration of two potentially important cellular markers of breast cancer outcome, M30 and Bcl-2, to detect CTC. They demonstrated that these markers can be monitored at baseline in patients with metastatic breast cancer, within a few days of initiation, and at first clinical follow-up after initiation of a new systemic therapy. The authors reported that 40% and 60% of CTC were apoptotic and expressed Bcl-2 at baseline, respectively, and that CTC levels at baseline were inversely related to the degree of apoptosis; they concluded that CTC-phenotyping through the fourth CellSearch® channel may predict clinical outcome beyond the mere counting of CTCs.

Hou et al¹⁴ reported the behavior of an integrated panel of cell death biomarkers (M30, M65, and nucleosomal DNA), using the fourth channel of CellSearch[®] in patients undergoing standard chemotherapy for small cell lung cancer, and concluded that both blood-borne cell death biomarkers and CTCs have the potential to enhance drug development as pharmacodynamic biomarkers in this tumor type.

The potentiality of CellSearch[®] in early stage cancer

Robust biological evidence on disseminated and CTC as surrogate for micrometastatic disease led to the introduction for the first time in the AJCC (American Joint Committee on Cancer) breast cancer staging manual (7th edition) of the cM0(i+) category, formally defined as deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other non-regional nodal tissue in the absence of clinical or radiographic evidence of distant metastasis.¹⁵ Therefore, after substantial confirmation of the prognostic and predictive value of CTCs in the metastatic setting, the detection and characterization in the early stage of cancer have become a major focus of translational cancer research. Detection of CTCs as a surrogate marker of early dissemination of cancer might have future application in early stage cancer patients for refining prognoses, monitoring response to treatment, and providing molecular characterization of residual disease after systemic therapy.

Few studies have investigated the clinical significance of CTC in non-metastatic colorectal cancer.^{16–18} Although preliminary results seem promising, no clear conclusion can be drawn from the published data due to the heterogeneity of study designs and CTC detection methods. The reported number of detectable CTCs in patients with stage I–III colorectal cancer is greatly variable, most likely due to the different detection methods (mainly immunological techniques and PCR [polymerase chain reaction]-based techniques) used in the studies. A prospective single-institution study published in 2008 by Sastre et al¹⁹ investigated whether a significant correlation exists between the presence of CTCs and clinic-pathological variables in colorectal cancer. A mixed population of 94 patients with early stage and metastatic colorectal cancer was enrolled. Blood samples were collected postoperatively, immediately before starting any adjuvant or palliative chemotherapy. The overall detection rate of CTC was 36.2%, and a significant correlation was demonstrated between the presence of CTC and the stage of disease, independently of the threshold used (≥ 2 CTCs per 7.5 mL of blood and ≥ 3 CTCs per 7.5 mL of blood). Particularly, the

presence of at least 2 CTCs in 7.5 mL of blood could be detected in almost a quarter of patients with stage II or III colorectal cancer, without significant differences between the two groups (20.7% in stage II, and 24.1% in stage III). In a second prospective study published in 2009 by the same research group, a heterogeneous large population of breast, colorectal, and prostate cancer patients at any stage was evaluated for the presence of CTCs.²⁰ Blood samples were collected again before the administration of any systemic therapy. Overall, 31.5% of patients had ≥ 2 CTCs per 7.5 mL of blood. The number of CTCs detected by the CellSearch[®] analysis was significantly higher (62.3% versus 14.0%) in metastatic patients compared with earlier stages of disease, and no differences were found among the three tumor types included in the analysis. A single-center study aimed to detect CTCs in the peripheral blood of patients with stage I–III colon cancer reported a 5% detection rate of ≥ 2 CTCs per 7.5 mL in preoperative samples from non-metastatic colorectal cancer patients.²¹ The authors further reported that none of the postoperative blood samples had CTC levels above the cutoff value. In a short report recently published by our own research group, a homogeneous population of high risk stage II or stage III colorectal cancer patients was prospectively evaluated for the presence of CTCs prior to adjuvant therapy.²² In this highly selected population of patients, CTCs were detected in 8 out of 37 (22%) patients. The study confirmed the significant correlation between the presence of CTCs and the stage of disease, and for the first time, to our knowledge, demonstrated a higher CTC detection rate in high-risk stage II colorectal cancer compared with low-risk stage II, suggesting that the detection of CTCs through CellSearch[®] in the non-metastatic setting of disease may allow the identification of stage II candidates for adjuvant chemotherapy and the selection of stage III patients for more- or less-aggressive approaches.

Globally, a relevant number of patients with early or locally advanced breast cancer were evaluated for the presence of CTCs in different studies. More than 2,000 patients were enrolled in the randomized Phase III SUCCESS study.²³ Nearly 22% of patients presented ≥ 1 CTCs per 23 mL of blood before the start of systemic treatment. The presence of CTCs before systemic treatment was an independent predictor of poor disease-free survival (DFS) and OS. Furthermore, the persistence of ≥ 1 CTC after adjuvant chemotherapy resulted in a decreased DFS, and the persistence of ≥ 5 CTCs was associated with decreased OS. Two recent studies reported the prognostic value of the presence of CTCs at the time of primary surgery in stage I–III breast cancer patients in a

series of 404 patients. Franken et al²⁴ reported the presence of ≥ 1 CTC in 30 mL before curative surgery in less than 20% of patients and demonstrated that the detection of CTCs preoperatively is associated with an increased risk for breast cancer-related death and DFS. Similarly, Lucci et al²⁵ reported that the presence of ≥ 1 CTC per 7.5 mL of blood, which was demonstrated in 73 of 302 (24%) patients, predicts early recurrence and decreased OS in chemo naïve patients with non-metastatic breast cancer. The prognostic impact of CTC detection in non-metastatic breast cancer patients has been extensively evaluated in the neoadjuvant setting. Two neoadjuvant Phase III German trials included the analysis of CTCs in their experimental design. In the GeparQuattro trial, the presence of ≥ 1 CTC per 7.5 mL was detected in 21.6% of patients.⁴ CTC detection did not correlate with primary tumor characteristics, and the decrease of CTC during treatment was not correlated with tumor response to neoadjuvant therapy. A similar detection rate (≥ 1 CTC per 15 mL of blood in 22.5% of patients) was demonstrated in a subanalysis of the Phase III neoadjuvant therapy GeparQuinto trial including 419 patients.²⁶ Significant decreases in CTC incidence and number per patient were observed during therapy, inversely to changes observed in circulating endothelial cell count, performed in parallel to CTC. The authors further confirmed the previously reported significant discrepancy between the HER2 status of CTCs compared with the corresponding primary tumor. The recently published updated results from the French REMAGUS02 study confirmed that the pre-chemotherapy detection of ≥ 1 CTC per 7.5 mL is associated with shorter distant metastasis-free survival and OS in breast cancer patients receiving neoadjuvant chemotherapy and that CTC detection has a negative impact on survival, even though this seems to be limited mainly to the first 3–4 years after the primary treatment.²⁷ Preliminary studies have aimed to investigate the potential prognostic value of CTCs in patients with early bladder cancer. Naoe et al,²⁸ who first investigated CTCs in urothelial cancer using CellSearch[®], reported CTC presence in 57.1% of patients with distant metastasis but no CTCs in patients with localized disease. More recently, Rink et al²⁹ published a prospective study aimed to investigate the biologic and clinical significance of CTCs in patients with clinically non-metastatic bladder cancer using CellSearch[®]. The authors found CTCs in 23% of patients and demonstrated that the presence of even a single CTC conferred a worse prognosis in terms of disease recurrence and cancer-specific and overall mortality. Similar results were obtained by our group in a selected population of non-muscle invasive bladder cancer patients, suggesting that in this very early setting of disease,

CTC counting through CellSearch[®] may allow the selection of patients with high risk of progression, and the identification of candidates for more aggressive treatments.³⁰

To date, small sample size is one of the major drawbacks of the studies aimed at investigating the prognostic and predictive significance of CTC counting in early stage cancer as well as in tumor types other than metastatic breast, colon, and prostate, where CellSearch[®] has not been validated for use in diagnostic procedure.

Clinical utility of CTCs: defining a midway state

So far, many clinical studies have focused on CTC counting in guiding prognosis in metastatic cancer patients. Nevertheless, much effort is still needed to answer the question of whether CTCs represent a potential surrogate marker for clinical endpoints.

Since the CellSearch[®] system was cleared by the FDA for CTC-based cancer diagnosis, its potential clinical utility is still to be fully demonstrated. So far, no large prospective studies have shown any predictive value for CTCs, and their clinical utility is therefore limited. The effect of the type of treatment on the prognostic and predictive value of CTCs has not been directly evaluated, and the ability of targeted therapies to modify the predictive value of CTC count has not yet been demonstrated. Furthermore, it is questionable whether results from trials lacking stratification of patients on the basis of molecular subtypes will be able to influence routine clinical practice specifically referring to the management of metastatic breast cancer patients.

On the other hand, the prognostic significance of CTC counting cannot be neglected. Bidard et al³¹ recently published the first European pooled analysis on clinical validity of CTC in 1,944 metastatic breast cancer patients treated between 2003 and mid-2012 in 17 centers. To date, this is the largest pooled analysis to assess the clinical validity of CTC count by the standardized CellSearch[®] technique. More than five CTCs per 7.5 mL at baseline were found in 47% of patients and were associated with shorter progression-free survival and OS. CTC changes after 3–5 and 6–8 weeks were also associated with progression-free survival and OS. Survival prognostications were significantly improved by adding CTC count at baseline to the clinicopathological models, while CEA (carcinoembryonic antigen) and CA15-3 (cancer antigen 15-3) levels at baseline and during therapy did not add further significant information.³¹

These results indicate that CTC counting may now be considered as having reached the highest level of evidence

for clinical validity in metastatic breast cancer patients for prognostic purposes.

Until CTCs are demonstrated as markers of early resistance to medical treatments, thus allowing an early switch of therapy with a demonstrated improved OS or DFS of patients, Heaven can wait.

Disclosure

The authors report no conflicts of interest in this work.

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