

Meeting Report

Circulating Tumor Cell Isolation and Diagnostics: Toward Routine Clinical Use

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Abstract

From February 7–11, 2011, the multidisciplinary Lorentz Workshop *Circulating Tumor Cell (CTC) Isolation and Diagnostics: Toward Routine Clinical Use* was held in Leiden (The Netherlands) to discuss progress and define challenges and potential solutions for development of clinically useful circulating tumor cell (CTC) diagnostics. CTCs, captured as "liquid biopsy" from blood, for counting and characterization using pathology and molecular assays, are expected to replace metastatic tissue biopsies to be used to predict drug response and resistance and to monitor therapy response and cancer recurrence. CTCs are highly heterogeneous; therefore, cancer type-specific isolation technologies, as well as complex clinical interpretation software, are required. *Cancer Res*; 71(18); 5955–60. ©2011 AACR.

Introduction

Since their first description, enumeration and characterization of circulating tumor cells (CTC) are thought to hold great promise (1). CTCs in blood originate from both primary and metastatic lesions, having acquired properties enabling migration into the circulation. A small subset of CTCs probably has the capability to establish metastatic growth after seeding in a tissue niche—supporting survival and clonal proliferation. These cells may have undergone epithelial–mesenchymal transition (EMT), acquired stem cell characteristics enabling self-renewal and creation of differentiated progeny, and may have become relatively resistant to standard chemotherapy. Whether, and where, a CTC succeeds in establishing a metastasis depends on its molecular profile, which is not necessarily the same as found in the primary tumor.

Driven by technical progress, both CTC enumeration and molecular characterization are expected to increasingly provide clinical information on prognosis, therapy choice, and effectiveness, as well as drug resistance, while also being of interest for drug development and biomarker discovery.

CTC diagnostics

In recent years, numerous assays to detect CTCs have been described. So far, the CellSearch system is the only

validated assay that has been cleared by the U.S. Food and Drug Administration as an aid to monitor patients with metastatic breast, prostate, and colon cancer. Using anti-EpCAM-coated magnetic beads, CTCs are extracted from blood, fixed/stained, and manually counted. The assay is prognostic in patients with metastatic breast, prostate, and colon cancer, and recently also non-small-cell lung carcinoma (NSCLC), and is being reimbursed by some insurance companies in the United States. Apart from counting, characterization of CTCs is of interest for companion diagnostic purposes (2). Aberrant functioning of specific intracellular signaling pathways drives cancer cell division and tumor growth. Many novel cancer drugs specifically target the responsible pathway—so-called targeted drugs. Consequently, assessment of whether or not a specific drug target is present in tumor cells becomes increasingly important. An example is trastuzumab, which inactivates the Her2/Neu pathway in breast cancer patients with overexpressed Her2/Neu protein. Unfortunately, targeted treatment inevitably leads to drug resistance, due to an alternative oncogenic signaling pathway taking over, requiring renewed tumor analysis to redefine (targeted) treatment. In line with this, in patients with metastatic Her2-positive primary cancer, following the first line of trastuzumab treatment, U.S. breast cancer guidelines require reassessment of Her2 status by tissue biopsy of a metastatic tumor. Unfortunately, this is not always possible and is often difficult and cumbersome. Moreover, metastases can differ from each other within one patient.

CTCs potentially provide a solution to circumvent this problem: Pathology and molecular analysis are expected to enable prediction of drug response, equivalent to a tissue biopsy. In line with this, using CellSearch, several pathology stainings (Her2, ER/PR, EGFR, AR, IGF1-R) are tested in clinical trials, and preliminary results indicate clinical utility (3–5).

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Challenges

Despite the promising results obtained so far, numerous important issues related to CTC definition, identification, isolation, diagnostics, clinical utility, and validation remain to be solved (6–8). During a 5-day workshop, 60 invited participants with clinical oncology, pathology, various diagnostic, engineering (including microfluidics and microfabrication), pharma, and regulatory backgrounds discussed how to define clinical utility, challenges, and potential solutions for CTC diagnostics (Fig. 1). Details on workshop presentations can be found at <http://www.lorentzcenter.nl/lc/web/2011/442/abstracts.pdf>. In the following text, conference presenters are referred to by name, with citations added for the related published work.

The Lorentz CTC Workshop: Facing the Challenges

The "true" CTC

CTCs in blood are very rare, with counts up to a few hundred per milliliter depending on available detection/isolation technology and CTC definition used, the latter being difficult in view of heterogeneity, presence of apoptotic CTCs, and circulating tumor fragments. Processing large sample volumes (1–30 mL blood) within an acceptable time frame, without damaging these vulnerable cells, is not trivial. Many CTCs currently may escape analysis, lacking a detectable characteristic such as EpCAM and cytokeratins, or because of damage or loss during the isolation process.

CTC heterogeneity between different cancers. Leon Terstappen and colleagues showed, using retrospective analysis of CTC images to define phenotypic CTC criteria, that different "CTC" definitions result in different CTC counts with varying degrees of clinical significance (9). Therefore, a "standard" CTC definition is clinically important. The CellSearch definition is considered the current standard: A nucleated circulating cell larger than 4 μm , expressing epithelial proteins EpCAM and cytokeratins 8, 18, and/or 19, while being negative for the leukocyte-specific antigen CD45. Many workshop contributions provided evidence for CTCs not obeying this definition, and the general consensus was that CTCs are heterogeneous and can differ between different cancers, as well as within one patient. No single parameter, such as size, cytomorphology, protein, or FISH biomarker staining, was considered essential or sufficiently distinctive to define the "true" CTC, each showing overlap with normal leukocytes and presence in normal volunteers. Combining parameters increases specificity, however at the cost of sensitivity (initials within parentheses in the text refer to presenters at the workshop, who are listed at the end of the article; LT, KP, JB, MM).

EpCAM is widely used in many CTC-detecting techniques. However, Sieuwerts and colleagues showed that breast cancer cell lines, specific subtypes, such as normal-like, lack EpCAM expression (10). CD146 may serve as an alternative marker to detect these EpCAM-negative cells, which frequently harbor stem cell characteristics, and a clinical study is ongoing to explore the clinical relevance of this CTC subset (11). CTCs

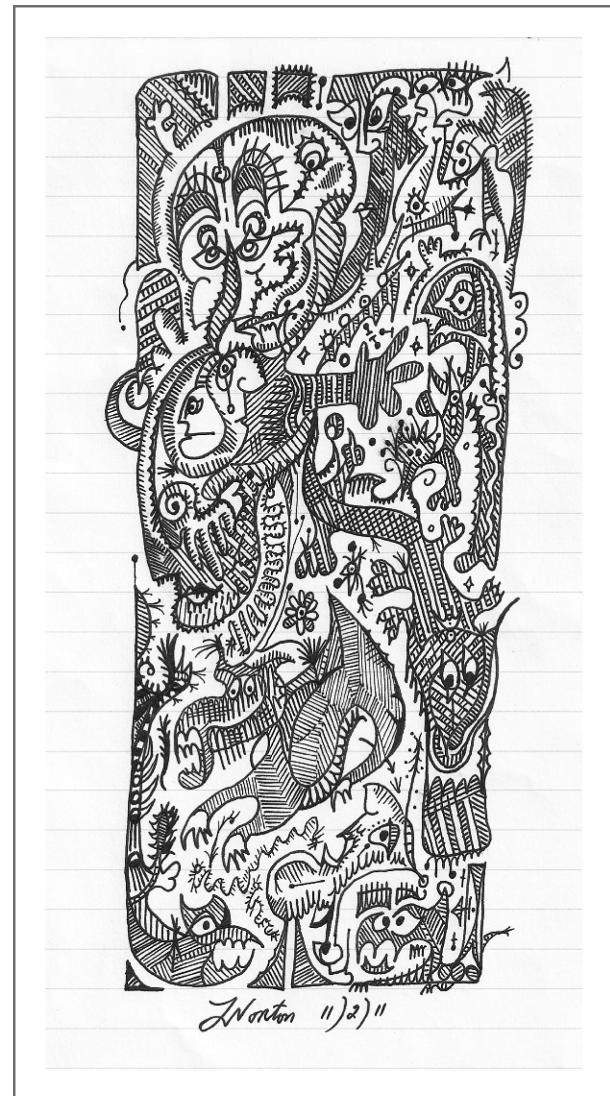


Figure 1. The Lorentz Workshop breathed a very relaxed, friendly, and scientific atmosphere, highly stimulatory for creative thinking, beautifully illustrated by this "doodle" drawing, created during one of the interactive sessions by Larry Norton from Memorial Sloan-Kettering Cancer Center in New York.

may also be detected using properties other than membrane markers, such as size. Matthew Krebs and colleagues presented data on ISET filter-enriched CTCs of lung cancer patients (12). Chwee Teck Lim and colleagues found EpCAM-negative CTCs, when selected on the basis of cell size and stiffness, for various cancer types (13). It is important to realize that there is currently no gold standard to determine the actual CTC count, that it is unlikely that one test will be able to detect all CTCs, and that CTC properties will differ across cancer types. In line, Marielena Mata suggested a CTC nomenclature/definition coupling CTCs to their cancer type of origin.

CTC heterogeneity within one patient. In addition to differences across cancer types, there is also heterogeneity

within individuals. In addition to EpCAM-positive, EpCAM-negative CTCs can be present, possibly influenced by disease stage, increasing in number with disease progression (JM, MK, JC, SJ, CL). EpCAM-negative CTCs can express stem cell markers such as vimentin, CD24/44, SNAIL/SLUG, ALD-1, and CD146, making them prime suspects for establishing metastases (ref. 10; JC, SJ, MK, JM, RK, LN). Furthermore, workshop participants presented evidence for cells expressing CD45 together with either Her2 (JM) or epithelial markers such as cytokeratin in a number of patients with different types of cancer (e.g., prostate, breast, head and neck; JB, JC, SJ, RK). Ruth Katz reported a high frequency of lung cancer-specific genomic rearrangements (FISH confirmed) in CD45-positive mononuclear cells in lung cancer patients. One can only speculate as to the meaning of CD45-positive cells with epithelial characteristics—could they be cancer stem cells differentiated along the hematopoietic lineage, or CTCs phagocytosed by leukocytes? The latter may be supported by the observation of mixed leukocyte/CTC-containing cell clusters (JC, SJ, RK, PK) and explain why live CTC counts diminish with time (SJ). Although some clusters have characteristic elongated forms, suggesting successful passing of capillary narrowings *in vivo*, clusters may also represent an *in vitro* artifact (SS); on the other hand, clusters may be lost during specific enrichment/isolation approaches (PK). If proven real, clusters could conceivably seed as micrometastasis in capillaries. Taken together, a CTC sample may be more heterogeneous than previously assumed.

Biological relevance

Larry Norton and colleagues showed how red and green fluorescent breast cancer cells, transplanted at different locations in a mouse, with time result in mixed red and green tumors, showing that aggressive CTCs migrate between tumors. Attracted by a combination of tissue and inflammatory factors (IL-6, IL-8), metastatic cells may repopulate the primary tumor (acting as a "sponge") or seed a recurrent tumor in breast tissue—the "self-seeding" hypothesis (14). After removal of the primary tumor, only locations outside the primary tumor niche are left to be seeded, which may explain why metastases expand following primary cancer surgery. On the basis of this hypothesis, a novel immunotherapeutic approach is being developed aiming at inducing an inflammatory response in the tumor to attract CTCs, which should act as immunogenic triggers for corecruited immune cells.

Jim Hicks and colleagues compared sequencing-based copy number variation profiles of single nuclei from different parts of a heterogeneous primary breast cancer to create a phylogenetic tree based on shared breakpoints. This approach revealed that *functional* cancer evolution takes "jumps" rather than proceeding gradually, with one cancer cell clone being largely responsible for populating both primary tumor and metastases (15). The remarkable similarity between complex genomic patterns observed in metastases and primary tumor is intriguing and compatible with the "self-seeding" hypothesis.

Clinical utility

Several prerequisites have to be met before an assay can be implemented into clinical care: for example, robustness, reproducibility, cost-effectiveness, but above all "clinical utility," meaning a clinically relevant decision can be taken based on assay result—this requires rigorous clinical validation (JB, KP, SS, LT, HS).

CTC enumeration for prognosis, recurrence, and therapy response monitoring. The clinical utility of the prognostic information provided by the CellSearch assay is still debated, having had no impact on treatment strategy yet, in part due to the disputed clinical meaning of CTC counts around the cutoff value. Future improvements may lie in adding exosome counting, present in 20-fold higher numbers, and by implementing algorithm-based feature recognition for automated counting which eliminates operator bias (16).

CellSearch is also used to *monitor* cancer recurrence/progression, as a patient friendly substitute for a regular CT scan (ML). On the basis of initial clinical trials, it moreover promises high clinical utility in monitoring therapeutic effectiveness. This is increasingly needed to rapidly terminate ineffective treatment to prevent side effects and unnecessary cost, for example with (neo)adjuvant and targeted therapy in breast cancer (KP).

CTC characterization for companion diagnostics. Given differences between primary and metastatic lesions, a significant number of patients with metastatic cancer may receive the wrong treatment when based on primary cancer tissue pathology (ref. 17; JM, HG). CTCs have potential clinical utility as a noninvasive, serial "liquid" biopsy to substitute for biopsies from metastases. For companion diagnostics, CTC morphology (18) and multiple pathology stainings for protein, RNA and DNA biomarkers are considered relevant, as well as genome (SJ, HS) and transcriptome (JM) analysis. In addition, CTC analysis may reveal novel companion diagnostic biomarkers that can be assayed for example as free DNA in blood (GC, EM).

CTC diagnostics for correct patient stratification during *early clinical trials* to prevent costly late drug failure is of high value for pharma—if sufficiently sensitive, validated, and against an acceptable price (GC, HG, KP, DS, RD, PL).

The ideal CTC companion diagnostics platform should reliably capture CTCs representative for all metastases present, in sufficient numbers and quality to conduct—and clinically interpret—companion biomarker assay(s), preferably on single-cell basis in view of CTC heterogeneity.

A "pathology-friendly" *imaging-based CTC characterization platform* may enable rapid clinical adoption (PK, DB), while not requiring CTC purification. Integration with Digital Pathology platforms, currently being developed to digitize, store, and process scanned images of pathology slides, may facilitate unbiased comparison between CTC and tissue pathology results (19). In addition to standard pathology stainings, *in situ* staining technologies for DNA/mRNA analysis, with single molecule resolution, are becoming available (20), further expanding possibilities of pathology diagnostics.

Isolation and careful handling of pure CTCs will be required for *molecular diagnostic characterization* (PCR, microarray, sequencing) on single CTC basis. Blood cells other than CTCs interfere with mRNA profiling, although restriction to epithelial-specific mRNAs may partially bypass this problem (21).

How to get hold of CTCs?

Considering CTC heterogeneity, requirements for a CTC assay will differ according to tumor type and are dependent on the ultimate goal: enumeration or characterization. The rarity and heterogeneity of CTCs define the challenge of isolating an extremely small number of CTCs from a huge amount of other cells in a large blood volume (typically ≥ 7.5 mL). Multiple different isolation approaches will probably be required, possibly consisting of combinations of less specific *enrichment* steps, followed by more specific *isolation* techniques (MK, PK, MM, SS).

Negative selection of non-CTCs may be useful to reduce sample volume; erythrocytes can be removed by selective lysis or filtration, and leukocytes by filtration or depletion by using antibodies not believed to be present on CTCs such as CD45 (ref. 22; DB, SJ, MK, BK, PG). All selection strategies carry a risk of introducing bias due to loss of CTCs. For this reason, a nonselective approach might be favored, for example by direct *high-definition* scanning and identification of CTCs among leukocytes on a substrate (18). An alternative approach, though at lower throughput yet, is *image flow cytometry* (23).

Positive CTC selection approaches are based on the premise that the targeted antigen is present on the CTCs, most commonly the EpCAM antigen as used by the CellSearch system. "MagSweeper" uses a similar approach with anti-EpCAM-coated magnetic beads dispersed in the sample and collected by sweeping magnetic rods (24). Microchip technologies from Harvard Medical School utilize microfluidic flow cells with either microposts or herringbone mixing channels (25) coated with EpCAM antibodies, generating flow patterns to optimize cell antibody contact while preserving cell viability. Brian Kirby and colleagues presented the "GEDI" microfluidic device containing PSMA (prostate-specific membrane antigen) antibody-coated obstacles arranged in patterns to create size-dependent particle trajectories favoring CTC capture and leukocyte rejection (26). Jean-Louis Viovy and colleagues used a microfluidic flow cell and antibody-coated magnetic beads, self-assembling into micropillars in an external magnetic field (27). The IMEC system is unique in integrating immunomagnetic bead capture with dielectrophoresis-based transport and a PCR assay (28). As EpCAM is not expressed by all tumor cells, future use of antibodies directed toward other markers, either alone or in mixture, is expected to yield more CTCs.

Isolation based on physical characteristics. Quite another approach is to separate CTCs on the basis of physical rather than biological characteristics such as size, stiffness (associated with a different cytoskeletal structure of cancer cells, compared with blood cells), or electrical properties.

Filtering techniques use difference in size and deformability of CTCs compared to blood cells, exemplified by the 8- μ m pore, pressure-driven, polycarbonate ISET filter (29) and 3-dimensional parylene microfilter containing homogeneous pores, presented by Ram Datar and colleagues, both enabling direct CTC visualization/analysis on the filter (30). Chwee Teck Lim and colleagues described their use of crescent-shaped microstructures (13) to efficiently capture viable CTCs, which by flow reversal can be retrieved, analyzed, and cultured—as shown for actual patient samples.

Dielectrophoresis can manipulate cells according to their dielectric properties in an inhomogeneous electrical field, which differ depending on size and membrane properties. Peter Gascoyne and colleagues developed a dielectrophoretic field-flow fractionation (DEP-FFF) device, enabling enrichment of viable CTCs (31). Hywel Morgan and colleagues built a microfluidic impedance cytometer for single-cell analysis to recognize CTCs based on differences in electrical impedance properties (32).

Finally, the EpiSpot technology, a functional cell culture assay based on counting CTCs by detection of secreted marker proteins, may be especially suited for drug development purposes (33).

Perspectives and Summary

Toward 2015, for (companion) diagnostics, one could envision a CTC characterization pathology platform enabling direct *in situ* comparison between CTC and tissue pathology, with smart algorithms for automated clinical interpretation, complemented later by single CTC DNA/RNA sequencing. Future CTC enrichment/isolation technologies may need to be tuned to specific (categories of) cancer types, whereas for drug development purposes, CTC culture presents an additional challenge.

While enabling repeated drug response prediction in patients with metastatic disease, CTC enumeration could be envisioned to monitor responses and recurrence. Adding CTC counting/diagnostics to early cancer diagnostics programs might improve the specificity of early detection and provide prognostic information.

To get there, in addition to novel CTC capture molecules, technologies are needed to deal with high sample volumes, and improve enrichment, purification, and characterization, using both physical and biological CTC parameters. Technologies should enable integration of various sample processing steps for subsequent diagnostic assays. Clinical interpretation requires complex software to deal with multiplex pathology staining or molecular assay results from a varying number of heterogeneous CTCs. Automated recognition of CTCs and objective staining quantification algorithms should be the standard, facilitating objective interpretation.

Enabling clinical decisions based on CTC analysis and obtaining regulatory clearance requires rigorous clinical trials. We conclude this report by stating that we believe that we speak for all workshop participants in saying that CTCs are on their way toward fulfilling their diagnostic promise in oncology.

Workshop Participants

Initials within parentheses in the text refer to presenters at the workshop, who are listed below: DB, David A. Basiji (Amnis Corporation, Seattle, Washington); JB, Johan De Bono (The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust, Sutton, United Kingdom); JC, Jeffrey Chalmers (Ohio State University, Columbus, Ohio); GC, Glen Clack (AstraZeneca Pharmaceuticals, Mereside, Macclesfield Cheshire, United Kingdom); RD, Ram Datar (University of Miami Leonard M. Miller School of Medicine, Miami, Florida); HG, Humphrey Gardner (Novartis Institutes for Biomedical Research, Cambridge, Massachusetts); PG, Peter Gascoyne (M.D. Anderson Cancer Center, University of Texas, Houston, Texas); SJ, Stefanie Jeffrey (Stanford School of Medicine, Stanford University, Palo Alto, California); RK, Ruth Katz (M.D. Anderson Cancer Center, University of Texas, Houston, Texas); BK, Brian Kirby (Cornell University, Ithaca, New York); MK, Matthew Krebs (Paterson Institute for Cancer Research, Manchester, United Kingdom); PK, Peter Kuhn (The Scripps Research Institute, La Jolla, California); PL, Phil Lefebvre (Abbott Molecular, Des Plaines, Illinois); CL, Chwee Teck Lim (National University of Singapore, Singapore); ML, Mine-tta Liu (Georgetown Comprehensive Cancer Center, Georgetown University, Washington, District of Columbia); JM, John

Martens (Erasmus Medical Center, Rotterdam, The Netherlands); MM, Marielena Mata (J&J, Raritan, New Jersey); EM, Evelyn McKeegan (Abbott Park, Illinois); LN, Larry Norton (Memorial Sloan Kettering Cancer Center, New York, New York); KP, Klaus Pantel (Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany); HS, Howard Scher (Memorial Sloan Kettering Cancer Center, New York, New York); DS, David R. Shalinsky (Pfizer, San Diego, California); SS, Stefan Sleijfer (Erasmus Medical Center, Rotterdam, The Netherlands); LT, Leon Terstappen (University of Twente, Enschede, The Netherlands).

Disclosure of Potential Conflicts of Interest

J.M.J. den Toonder and A. van de Stolpe: employment, Philips Research. K. Pantel: honoraria, Veridex, Novartis, AMGEN, Bayer, Prometheus, Cell Signaling Technologies, BD, and Roche. S. Sleijfer: research funding, J&J. L.W. Terstappen: scientific advisor, Veridex (J&J).

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