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Abstract
Hematologic spread of carcinoma results in incurable metastasis; yet, the basic characteristics and travel mechanisms of cancer cells in the bloodstream are unknown. We have established a fluid phase biopsy approach that identifies circulating tumor cells (CTCs) without using surface protein-based enrichment and presents them in sufficiently high definition (HD) to satisfy diagnostic pathology image quality requirements. This 'HD-CTC' assay finds >5 HD-CTCs mL^{-1} of blood in 80% of patients with metastatic prostate cancer ($n = 20$), in 70% of patients with metastatic breast cancer ($n = 30$), in 50% of patients with metastatic pancreatic cancer ($n = 18$), and in 0% of normal controls ($n = 15$). Additionally, it finds HD-CTC clusters ranging from 2 HD-CTCs to greater than 30 HD-CTCs in the majority of these cancer patients. This initial validation of an enrichment-free assay demonstrates our ability to identify significant numbers of HD-CTCs in a majority of patients with prostate, breast and pancreatic cancers.

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Introduction

The field of circulating tumor cell (CTC) research has evolved rapidly in response to a significant unmet medical need for longitudinal disease monitoring in patients with epithelial cancers (carcinomas). Predicting and monitoring therapy response and disease progression are particularly important due to changes in the therapy-responsiveness of disease over the course of a patient's cancer. In liquid tumors such as leukemias, the malignant cells can be easily sampled from the bloodstream at many points during the disease, and appropriate therapy adjustments applied. However, solid tumors such as carcinomas are generally sampled only at the time of initial diagnosis, as tissue biopsies are invasive procedures with known risks. Occasionally, a repeat tumor sampling is collected at the time when distant metastasis first becomes apparent, to confirm that distant lesions in fact represent metastases from the patient's known primary tumor. Thus, in our understanding of cancer behavior, while progress has been made in understanding the solid tissue forms of primary and metastatic carcinomas in their respective microenvironments, a substantial gap exists in understanding carcinoma behavior during the fluid phase, wherein it occupies and spreads via the bloodstream. For cancers that occur predominantly as solids, the elusive and scant circulating component contains within it the cells giving rise to future distant metastases, and as such, represents a compelling target for investigation.

Research to fully characterize the clinical significance of this fluid phase of solid tumors has been hindered by the lack of easily accessible and reliable experimental tools for the identification of CTCs. The unknown character and low frequency of CTCs in the blood, combined with the difficulty of distinguishing between cancerous versus normal epithelial cells, have significantly impeded research into how the fluid phase might be clinically important. The ideal fluid phase biopsy would find significant numbers of CTCs in most epithelial cancer patients, and would preserve and present CTCs to a diagnostic pathologist and/or researcher in a format that enables not only enumeration but further molecular, morphologic and/or phenotypic analysis. In addition, it should preserve all other CTC-like cell populations within the sample for further analysis.

Currently, the only FDA-approved technology for CTC detection is based on immunomagnetic enrichment. This current 'gold standard' test is called CellSearch® and employs an immunomagnetic enrichment step to isolate cells that express the epithelial cells' adhesion molecule (EpCAM) [1]. Additionally, to be identified as a CTC, the cell must contain a nucleus, express cytoplasmic cytokeratin, and have a diameter larger than 5 μm. This technology has uncovered the prognostic utility of enumerating and monitoring CTC counts in patients with metastatic breast, prostate, and colorectal cancers; however, the sensitivity of this system is low, finding no or few CTCs in most patients [2, 3]. Most follow-on CTC technologies have reported higher sensitivity and are pursuing variations of the enrichment strategy; however, these approaches directly bias the detectable events toward those that have sufficient expression of the protein selected for the initial enrichment step [4–8].

The approach used herein to identify CTCs in high definition (HD-CTCs) is distinct in that it does not rely on any single protein enrichment strategies. Instead, all nucleated cells are retained and immunofluorescently stained with monoclonal antibodies targeting cytokeratin (CK), an intermediate filament found exclusively in epithelial cells, a pan leukocyte specific antibody targeting CD45, and a nuclear stain, DAPI. All nucleated blood cells are imaged in multiple fluorescent channels to produce high quality and high resolution digital images that retain fine cytologic details of nuclear contour and cytoplasmic distribution. This enrichment-free strategy results in high sensitivity and high specificity, while adding HD cytomorphology to enable detailed morphologic characterization of a CTC population known to be heterogeneous. A key advantage of this approach is that multiple analysis parameters can be pursued to identify and characterize specific populations of interest.

Using this approach, we have previously identified, morphologically characterized, and credentialed CTCs in relation to their primary tumors, in case studies of breast, colorectal, and lung cancer patients. In brief, through morphologic examination of CTCs in a patient with a well differentiated lung adenocarcinoma, we identified circulating cells with morphologic features consistent with this type of tumor, including, for example, cells with relatively low nuclear-to-cytoplasmic ratios. The morphology of the tumor cells identified in circulation mimicked the morphology found in that patient's fine needle aspirate biopsy of the primary tumor [9]. Evaluated in a larger cohort of breast and colorectal cancer patients the CTCs exhibited a high degree of inter- and intra-patient heterogeneity in cytologic appearance consistent with the morphologic heterogeneity of cells commonly found in the primary and metastatic tumor [10, 11].

We now report on assay results demonstrating 'HD-CTCs' in a small cohort of metastatic cancer patients. The key innovative aspects of this assay are its simplicity, with minimal processing to the blood specimen, and its ability to enable professional morphologic interpretation with diagnostic pathology/cytopathology quality imagery.

The data presented here demonstrate the first use of the HD-CTC assay in a controlled prospective protocol which addressed the reliability and robustness of the assay, compares sensitivity in a split sample comparison with the CellSearch® assay, and establishes the incidence of HD-CTCs and HD-CTC clusters (see publication in this issue of Physical Biology) in patients with metastatic breast, prostate, and pancreatic cancers as well as normal controls. Importantly, the definition of 'HD-CTC' is strict and requires that the cell(s) have an intact nucleus, express cytokeratin and not CD45, be morphologically distinct from surrounding benign white blood cells (WBCs), and have cytologic features consistent with intact morphologically abnormal epithelial cells suitable for downstream analysis.

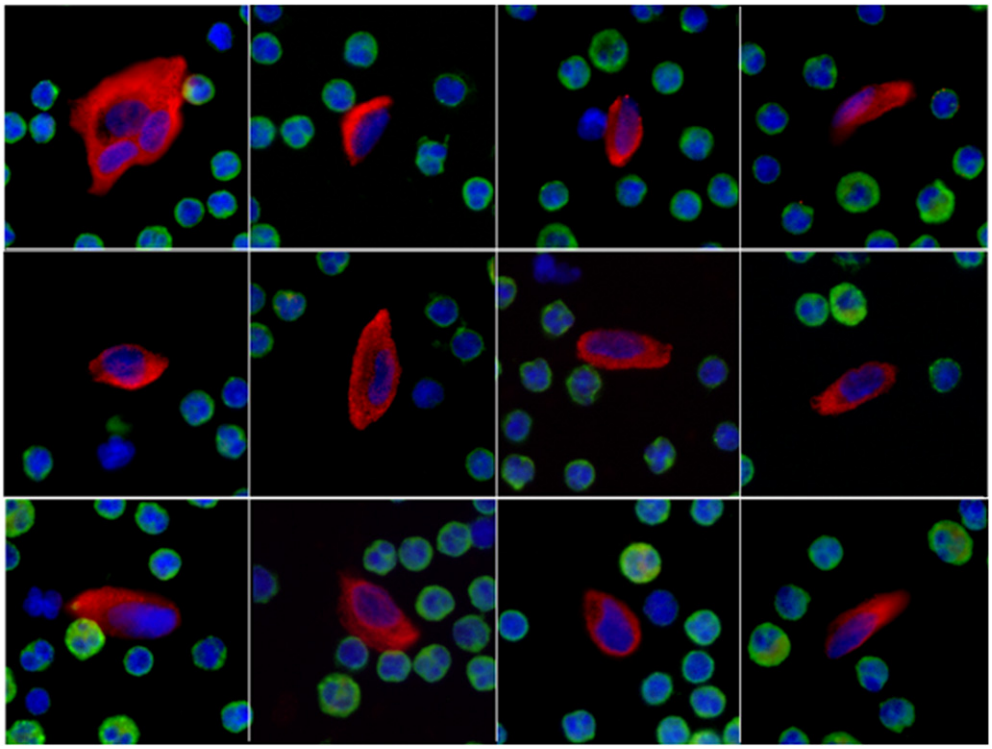

Figure 1. Gallery of representative HD-CTCs found in cancer patients. Each HD-CTC is cytokeratin positive (red), CD45 negative (green), contains a DAPI nucleus (blue), and is morphologically distinct from surrounding WBCs.

Results

CTC to HD-CTC: definitional refinements

Our previous work has established a definition of an intact CTC based on the investigation of large numbers of candidate events in patients with epithelial cancers, with direct comparison to cell from the solid forms of the same tumor in the same patient [9–12]. Based on these definitions, and utilizing the HD-CTC assay to refine criteria, a definition of an HD-CTC was established. This definition has been developed to ensure that an HD-CTC is a cell that has the highest potential of being an intact cell originating from a solid deposit of carcinoma in the patient's body. All other populations that partially fulfill these requirements but fall short of the strict inclusion criteria discussed below are tracked in the analysis since many of them likely represent fragmented or apoptotic tumor cells that might have biologic significance [13], but are excluded in the HD-CTC count because of their unpredictable suitability for evaluation by further downstream methodologies.

This platform allows for simultaneous cytomorphologic review of fluorescent images with individual channel images, augmented with cell-by-cell annotation with ancillary semi-quantitative data regarding size and fluorescent intensity of objects. HD-CTCs are classified as cells that are (a) cytokeratin positive, and (b) CD45 negative, with an intact non-apoptotic appearing nucleus by DAPI imaging. Positivity for CK is defined as the fluorescent signal being significantly above the signal of surrounding cells. Negativity is defined as being the same level or below the signal of the surrounding cells. Negativity for CD45 is defined as having intensity below visual detection under the boundary condition that 99% of all cells are detectable globally. A gallery of representative HD-CTCs is displayed in figure 1.

Mild apoptotic changes in the cytoplasm are accepted, such as cytoplasmic blebbing visualized in the cytokeratin channel as long as the nucleus does not appear apoptotic. In addition to these semi-quantitative characteristics, HD-CTCs must be morphologically distinct from normal WBCs, and must have a morphology that is compatible with a malignant cell by criteria used in standard diagnostic cytopathology, predominantly embodied as enlarged size, but also encompassing cytomorphologic features such as architectural organization of nucleus and cytoplasm, cytoplasmic shape, and nuclear shape. We have established a lower nuclear size cut-off of 1.3 times the mean WBC nuclear size. Although somewhat arbitrary, as no modern consensus has been established and the biologic truth is as yet unknown, we selected this limit based on evaluation of the largest nuclear size of cells identified as WBCs in healthy donors showing false nonspecific staining with cytokeratin (i.e. CD45 positive and cytokeratin positive). As virtually all viable epithelial cells are larger than virtually all leukocytes in routinely fixed and stained human tissues across the clinical spectrum of cancer diagnosis, this approach is felt to be a conservative assumption. At the other end of the spectrum, a common morphologic feature of HD-CTCs is larger nuclei up to five times the average size of surrounding WBC nuclei. Other commonly observed features include nuclear contours distinct from those of surrounding WBC nuclei (e.g. elongation), large cytoplasmic domain with an eccentric distribution of cytoplasm relative

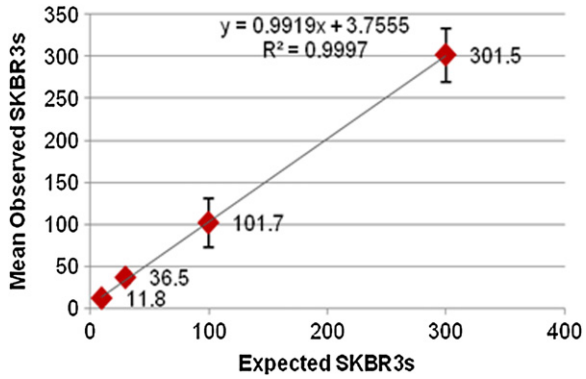

Figure 2. Mean observed SKBR3s plotted against expected SKBR3s. Four aliquots of normal control blood was spiked with varying numbers of SKBR2 cells to produce four slides with approximately 10, 30, 100, and 300 cancer cells per slide. The mean of each quadruplicate is displayed as well as error bars noting standard deviation.

Table 1. Percentage of patients with HD-CTCs per 1 mL of blood obtained from metastatic prostate, breast, and pancreatic cancer patients as well as normal controls.

	N	⩾2	⩾5	⩾10	⩾50
Prostate	20	90%	80%	65%	40%
Breast	30	80%	70%	60%	27%
Pancreatic	18	61%	44%	44%	11%
Normal	15	7%	0%	0%	0%

to nucleus, polygonal or elongated cytoplasmic shape, and frequent doublets and clusters of three or more HD-CTCs.

Incidence of HD-CTCs in patients with metastatic cancer

HD-CTCs were enumerated in a cohort of 30 metastatic breast cancer patients, 20 metastatic prostate cancer patients, 18 metastatic pancreatic cancer patients, and 15 normal controls. The incidence of HD-CTCs in the three types of cancers investigated is displayed in table 1. Using this approach, ⩾5 HD-CTCs mL^{-1} were found in 80% of the prostate cancer patients (mean = 92.2), 70% of the breast cancer patients (mean = 56.8), 50% of the pancreatic cancer patients (mean = 15.8), and 0% of normal controls (mean = 0.6).

Assay linearity and sensitivity using spike-in experiments

To test assay linearity and sensitivity, various numbers of breast cancer cell line SKBR3s were spiked into normal control blood in quadruplicates and processed according to the HD-CTC assay. As displayed in figure 2, mean observed SKBR3s are plotted against expected SKBR3s and display a correlation coefficient (R^2) of 0.9997.

Assay robustness of HD-CTC counts in patients with carcinomas

The assay robustness of the HD-CTC assay was tested against multiple processors and split samples. Duplicate tests were performed by two separate processors on nine different patient samples. Figure 3 displays a comparison of HD-CTC mL^{-1} counts between two processors using split samples giving a regression equation for this comparison of $Y = 1.163x - 4.3325$ with a correlation coefficient (R^2) of 0.979. All data were analyzed by a single operator blinded to the experiment. The slope of the line is greater than 1 suggesting a slight systematic linear variation between the two processors.

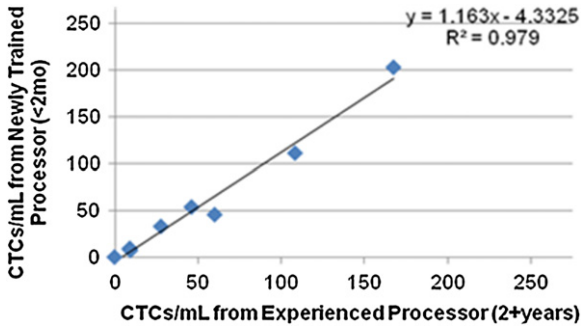

Figure 3. Comparison of CTC counts between two separate processors on nine different cancer patient samples. CTC mL^{-1} counts ranged from 0 to 203.

Assay specificity in samples from normal controls

Fifteen healthy donors from an institutional healthy donor pool were evaluated as a control population consisting of 8 females and 7 males with an age range of 24 to 62 years. In all but one healthy control, the number of such events when corrected for volume was 1 HD-CTC mL^{-1} or less. The outlier was a healthy female donor with an HD-CTC count of $4/mL^{-1}$. Upon explicit re-review of each of her cells, about one third of them easily met all inclusion criteria, while the remaining two thirds fulfilled all criteria but were near the lower limit for inclusion by one or more criteria. Four other healthy donors fell into the non-zero category, with 1 HD-CTC mL^{-1} each. Explicit re-review of these cells revealed a similar pattern, in that about one third strongly met all criteria, while the remaining two thirds of the cells fulfilled criteria, but were near the lower limit for inclusion by one or more criteria. Examples of the latter type of event include cells that measure 30% larger than surrounding WBCs but do not appear significantly larger by morphologic evaluation, and cells that are slightly out of focus and might have apoptotic nuclear changes that are not detectable by eye, and finally, occasional cells that have objective cytokeratin intensity measurements above the cut-off but subjectively do not appear significantly brighter than surrounding WBCs by single channel fluorescent review.

Comparison of HD-CTC assay to CellSearch®

A total of 15 patients (5 metastatic breast cancer and 10 metastatic prostate cancer) were evaluated for CTCs with both CellSearch® and the HD-CTC assays. Two tubes of blood were collected from each patient. One tube of 7.5 mL of blood was collected in CellSave tubes (Veridex, Raritan, NJ)

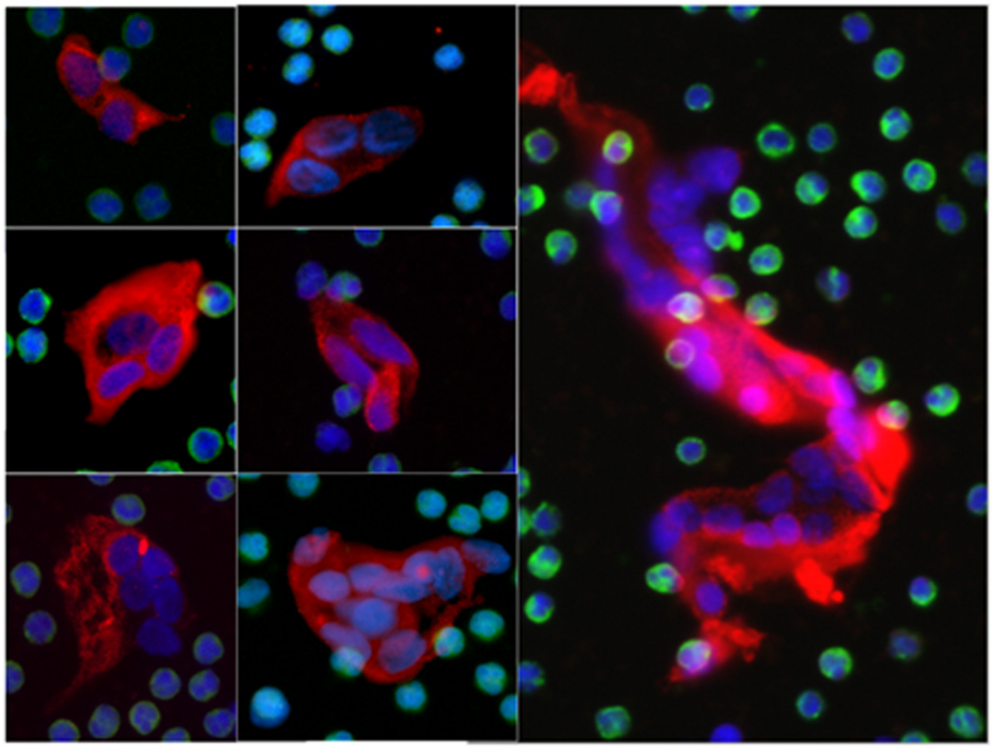

Figure 4. Gallery of representative clusters that are found in most patients with cancer. Clusters range from 2 to over 30 HD-CTCs. Each HD-CTC is cytokeratin positive (red), CD45 negative (green), contains a DAPI nucleus (blue), and is morphologically distinct from surrounding nucleated cells.

Table 2. Comparison of HD-CTC assay to CellSearch from five metastatic breast cancer patients and ten metastatic prostate cancer patients. CellSearch values are extrapolated to number of CTCs per mL of blood.

Cancer type	HD-CTCs mL^{-1}	CellSearch/mL
Breast #1	49.3	0.1
Breast #2	87	0
Breast #3	33.4	0.1
Breast #4	199.3	0.1
Breast #5	5	3.1
Prostate #1	2.3	0
Prostate #2	8.4	0.4
Prostate #3	107.3	2.8
Prostate #4	1.3	0
Prostate #5	150.5	0.1
Prostate #6	0	0
Prostate #7	1.4	0.5
Prostate #8	1.5	0.1
Prostate #9	145.3	0.8
Prostate #10	57.6	0

and sent to Quest Diagnostics (San Juan Capistrano, CA) for enumeration of CTCs using the CellSearch® assay. A second tube of blood was collected from each patient and processed according to the HD-CTC protocol 24 h after the blood draw, consistent with the standard HD-CTC process in order to mimic the timing at which samples were processed at Quest Diagnostics. Table 2 shows the results from this side by side comparison. The CellSearch® assay detected two or more CTCs per 7.5 mL of blood in 5/15 patients tested. In contrast, the HD-CTC assay detected significantly higher numbers of CTCs in significantly more patients (HD-CTCs were identified in 14/15 patients tested).

Morphologic range of HD-CTCs

We found a morphologically heterogeneous population of HD-CTCs within and across patients.

HD-CTCs had various shapes, sizes, and cytokeratin intensities. In some cases, distinctive cytologic features such as large size or polygonal cytoplasmic shape were quite distinctive and monotonous within the patient's sample. In other cases, there was cytomorphologic variability between HD-CTCs within a single sample. Cell size also varied; many patient samples had HD-CTCs with nuclei uniformly three or four times the size of neighboring WBC nuclei, while other patients had cells with nuclei uniformly only 1.3 times the size of neighboring WBC nuclei. Some patients had a range of sizes.

Interestingly, using this platform that allows for detailed morphologic evaluation, HD-CTC doublets and clusters were identified in the majority of the cancer patients in this cohort (88%), ranging from clusters of 2 HD-CTCs to greater than 30 HD-CTCs. Figure 4 shows examples of typical clusters.

Morphology of 'other' cell types

Other cell-like objects that are cytokeratin positive, CD45 negative, and contain a nucleus but do not meet the inclusion criteria are not counted as HD-CTCs but are tracked by the assay. The purpose of this approach is to have strict

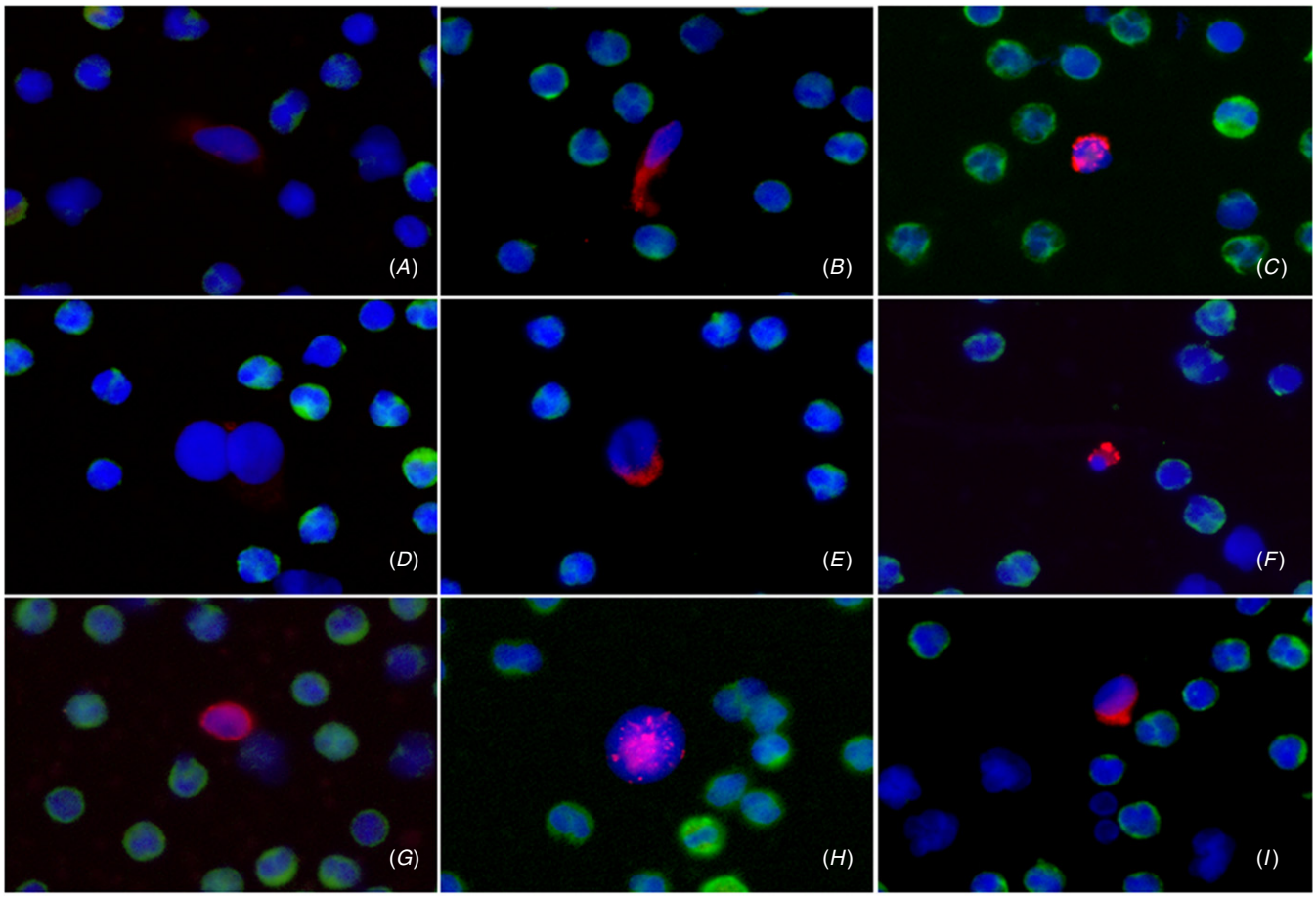

Figure 5. Gallery of candidate HD-CTCs that were excluded because they lacked various morphologic or morphometric inclusion criteria. (*A*) Cytokeratin intensity too dim. (*B*) Nuclear size too small. (*C*) Cytokeratin insufficiently circumferential (surrounds less than two thirds of nucleus). (*D*) Cytokeratin too dim, although appears to be a cluster of two very large cells. (*E*) Nucleus shows apoptotic disintegration changes. (*F*) Nucleus too small and cytoplasm insufficiently circumferential; appears to be a cell in late apoptosis. (*G*) Nucleus too small (same size as surrounding WBC nuclei). (*H*) Cytokeratin present, but not circumferential. (*I*) Cytoplasm insufficiently circumferential, nucleus too small.

inclusion/exclusion criteria for a specific intact phenotype of CTCs, while retaining access to objects that only partially fulfill such criteria, yet might still be clinically meaningful, such as apoptotic tumor cells, tumor cell fragments, or cells undergoing epithelial to mesenchymal transition [13].

Thus, in addition to tracking HD-CTCs, a number of different categories of cytokeratin positive cells were cataloged in this cohort of patients, including cells that had nuclei displaying apoptosis, cells that did not have circumferential cytokeratin, cells that were the same size or smaller than surrounding WBC, and cells that were cytokeratin dim or negative. Representative cells that were tracked but not called HD-CTCs are shown in figure 5. Finally, in addition to various types of bright cytokeratin positive cells, many patients had a substantial number of cells with nuclei that were morphologically distinct from surrounding WBC, resembled the nuclei of the HD-CTCs within that sample, and were CD45 negative, but were also cytokeratin dim or negative (figure 6).

In light of the extensive current debate about the possible existence of carcinoma cells undergoing epithelial-to-mesenchymal transition, the appearance and protein expression pattern of these cells identify them as possible candidates for such a cell type. Alternatively, these cells could be older tumor cells that have been stripped of most of their cytoplasm.

Discussion

Fluid biopsy analysis holds the promise of revealing metastasis in action. Within this elusive cell population are the cancerous seeds that spread through the bloodstream and lead to eventual distant metastases. But in order to interrogate them and apply the findings clinically, the cells must be reliably recoverable in the majority of cancer patients. We describe here a maximally inclusive, minimally destructive, yet cytologically selective platform that yields high quality cells in HD in high numbers of cancer patients. Initially noted as a rare occurrence in prostate cancer patients [13], for the first time we identify clusters of these cells in the majority (88%) of patients with metastatic cancer.

The incidence of CTCs using our assay is much higher than that reported with many technologies and is in the same

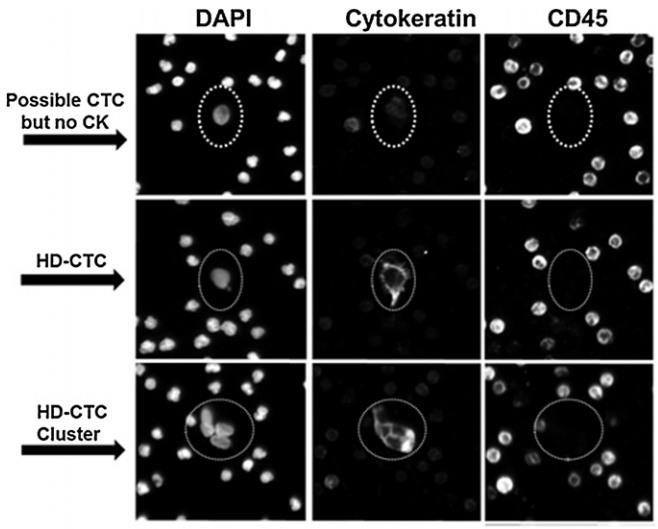

Figure 6. Representative images of types of suspected CTCs found in a single prostate cancer patient. Top row: suspected CTC that is negative for cytokeratin and CD45, but has a nucleus that is large and looks like other HD-CTCs found in this patient. Middle row: typical HD-CTC that is cytokeratin positive, CD45 negative, with a DAPI nucleus. Bottom row: HD-CTC cluster of four cells.

range as reported by the CTC-chip [6]. Additionally, a direct comparison to CellSearch® showed significantly more CTCs detected by the HD-CTC assay, in a higher proportion of patients. In addition to higher sensitivity, the assay also demonstrates robust performance in both cell lines and patient samples. The reproducibility and robustness of the assay with a semi-quantitative characterization of each event are critical for our interest in downstream analysis of cells.

Morphologically, we found a heterogeneous population of CTCs within and across patients. CTCs had various shapes, sizes, and cytokeratin intensities. In some cases, distinctive cytologic features such as large size or polygonal cytoplasmic shape were quite distinctive and monotonous within the patient's sample. In other cases, there was cytomorphologic variability between HD-CTCs within a single sample. Cell size also varied inconsistently; many patient samples had HD-CTCs with nuclei uniformly three or four times the size of neighboring WBC nuclei, other patients had cells with nuclei only a third again as large as neighboring WBC nuclei, and other patients had high intra-patient size variability (data not shown.)

Surprisingly, our cohort of patients demonstrated the common occurrence of clusters of HD-CTCs in most patients. 88% of the metastatic cancer patients evaluated in this cohort study showed clusters ranging in size from 2–30 HD-CTCs. Multiple questions arise around the presence of such clusters, including the rheology of transit through the circulatory system of such a large aggregate, as well as biological questions about whether such clusters represent 'tumorlets' that are transporting their own microenvironmental stroma with them as they travel and thus may be the most, or only, truly metastable CTCs. Current investigations are underway to further characterize the clusters in this cohort of patients.

In addition to enumerating HD-CTCs, various other categories of CTC-like cells were independently tracked, including cells that had nuclei displaying apoptosis, cells lacking circumferential cytokeratin, cells that were the same size or smaller than surrounding WBC, and CD45-negative cells that were cytokeratin dim or negative (figure 5). Although many of these events may in fact represent circulating malignant epithelial cells in various stages of biologic anoikis or mechanical disruption secondary to even the minimal processing utilized in the platform, others likely represent false positives of various types. Our initial goal is to identify a population of cells with a very high likelihood of including all potentially metastasizing epithelial cells that are suitable for downstream analysis by secondary methodologies. Fragmented, disrupted, pyknotic, or otherwise damaged carcinoma cells are not considered reliable for secondary analysis in standard diagnostic pathology, and thus we exclude them for purposes of 'counting viable CTCs' in this fluid phase biopsy platform as well. We do, however, locate, enumerate and track them, as we recognize that their presence likely correlates overall with the tumor biology in the patient, either by reflecting overall tumor burden or by reflecting some as yet ill-understood complex equation involving tumor burden and tumor vascularity and efficiency of intravascular immune surveillance.

One of the interesting non-HD-CTC categories, often seen in patients who have HD-CTCs elsewhere on the slide, consists of cells with nuclei that are morphologically distinct from surrounding WBC, generally by size criteria, and were CD45 negative, but are also cytokeratin dim or negative. As one of the most significant advantages of the HD-CTC assay is that parallel aliquots of cells are frozen, allowing for retrospective marker selection in specific high-yield patient samples, ongoing studies to further characterize such cells are in progress. Possibilities include epithelial cells with denatured or stripped cytoplasm, cells aberrantly expressing or aberrantly lacking proteins typical for their biologic origin, or possibly cells undergoing a metaplastic process such as epithelial-to-mesenchymal transition. The assay and imaging platforms are currently limited to analysis of fixed cells; however efforts are underway to establish the potential utility of this approach for live cell enumeration and imaging.

While the sample sizes of our respective patient cohorts are still too modest to draw any firm conclusions, it is noteworthy that the frequency of detection, and relative concentration, of CTCs among different tumor types using our approach (prostate > breast > pancreatic) parallels the findings observed using other methods such as CellSearch. Previous investigators have suggested that biologic or anatomic differences in tumor vascularization, the anatomic sites of metastasis, and whether tumor cells are filtered via the portal circulation may account for some of these differences [1].

Pitfalls abound in the field of CTC biology. Among the most difficult issues are sensitivity and specificity. The rate of true biologic positives in cancer patients is unknown, and the rate of circulating benign epithelial cells in healthy people or people with non-malignant disease is likewise not known with certainty. For sensitivity—a positive test in the presence

of disease (in this case, the biologic presence of CTCs)—the strategy commonly used in place of sure knowledge is either spiking experiments placing cell line cells into whole blood, or else published data from other researchers using technologies that may vary significantly. Both approaches are problematic and the issue persists in the field. Specificity—a negative test in the absence of disease—can be addressed at least partially by evaluating patient samples that, according to our current understanding of cancer, 'should' be negative for circulating cancer cells. To this end, healthy donor blood is generally used as a 'negative control', and while published numbers vary, in general only low numbers of CTCs are found in clinically healthy people [1]. In our study, the healthy donor population consists of non-laboratory member volunteers of varying ages who are not known to have cancer but who have not undergone extensive medical evaluations for occult cancers. Since all cancers are, of course, initially asymptomatic, finding a truly negative population for specificity determination would be an expensive and long term effort, as one would need to conduct either invasive medical testing on the apparently healthy subjects, or else wait a sufficient amount of time to be sure that they do not manifest some type of carcinoma over subsequent years.

In our control population, we did observe >1 HD-CTCs mL^{-1} in one healthy control. Does this healthy volunteer have occult carcinoma? The answer is unknown at present. This further raises the general issue of HD-CTC definitions in the two very different settings of screening (i.e. finding cancer-like events in apparently healthy people) versus tumor marker evaluation (i.e. finding cancer-like events in people who are known to have cancer.) Eventually, the cut-off for clinical significance will be different in these two patient populations, based on expected incidence of disease. This study was not designed as a screening study, and thus the findings in healthy donors are presented and morphologically described in detail here not to suggest a clinically useful screening strategy for a population of healthy people, but rather as an informative contribution to the discussion of circulating cytokeratin positive events in cancer and non-cancer patients. Finally, we consider that in the metastatic cancer patient population, depending on the question, the appropriate control population is likely actually a cohort of non-metastatic cancer patients with the same primary tumor type, but because this control group can only be identified retrospectively when they have failed to recur after years of follow up, determination of the true specificity for any CTC assay is a long term endeavor.

In summary we have provided data that the HD-CTC assay (i) finds significant number of CTCs in most patients with metastatic cancer, (ii) has improved sensitivity over the CellSearch system, (iii) provides HD-CTCs in an ideal format for downstream characterization, and (iv) enables the prospective collection of samples that can be stored frozen for long periods of time and then retrospectively analyzed as new assays or markers become available.

Methods

Patients and blood sample collection

Samples were collected from metastatic cancer patients in anti-coagulated blood tubes at Scripps Clinic; University of California, San Diego; Billings Clinic; and University of California, San Francisco under IRB approved protocols. Samples from non-local sites (UCSF, Billings Clinic) were shipped overnight so that the sample was received and processed within 24 h. Samples from local sites (Scripps Clinic and UCSD) were held at room temperature for 16–24 h to mimic samples coming from non-local sites. Blood specimens were also drawn from normal controls from the TSRI Normal Blood Donor Service.

Blood sample processing for HD-CTC detection

Blood specimens were rocked for 5 min before a WBC count was measured using the Hemocue WBC system (HemoCue, Sweden). Based upon the WBC count, a volume of blood was subjected to erythrocyte lysis (ammonium chloride solution). After centrifugation, nucleated cells were re-suspended in PBS and attached as a monolayer on custom made glass slides. The glass slides are the same size as standard microscopy slides but have a proprietary coating that allows maximal retention of live cells. Each slide can hold approximately three million nucleated cells; thus the number of cells plated per slide depended on the patients' WBC count.

For HD-CTC detection in cancer patients for this study, four slides are used as a test. The remaining slides created for each patient are stored at -80 °C for future experiments. Four slides were thawed from each patient, then cells were fixed with 2% paraformaldehyde, permeabilized with cold methanol, and non-specific binding sites were blocked with goat serum. Slides were subsequently incubated with monoclonal anti-pan cytokeratin antibody (Sigma) and CD45-Alexa 647 (Serotec) for 40 min at 37 °C. After PBS washes, slides were incubated with Alexa Fluor 555 goat anti-mouse antibody (Invitrogen) for 20 min at 37 °C. Cells were counterstained with DAPI for 10 min and mounted with an aqueous mounting media.

Imaging and technical analysis

All four slides from each patient were scanned using a custom made fluorescent scanning microscope which has been developed and optimized for fast, reliable scanning. Each slide was scanned entirely at $10\times$ magnification in three colors and produced over 6900 images. The resulting images were fed to an analysis algorithm that identifies likely candidate HD-CTCs based upon numerous measures, including cytokeratin intensity, CD45 intensity, as well as nuclear and cytoplasmic shape and size. A technical analyst then goes through algorithm generated likely candidates and removes hits that are obviously not cells, such as dye aggregates.

Professional analysis and interpretation

All likely candidate CTCs are presented to a hematopathologist for analysis and interpretation through a web-based report where the pathologist is able to include or exclude each candidate cell as an HD-CTC. Cells are classified as HD-CTCs if they are cytokeratin positive, CD45 negative, contained an intact DAPI nucleus without identifiable apoptotic changes (blebbing, degenerated appearance) or a disrupted appearance, and are morphologically distinct from surrounding WBCs (usually a shape-based feature, although occasionally purely size based.) They must have cytoplasm that is clearly circumferential and within which the entire nucleus is contained. The cytoplasm may show apoptotic changes such as blebbing and irregular density or mild disruption at the peripheral cytoplasmic boundary, but must not be so disrupted that its association with the nucleus is in question. The images are presented as a digital image, with individual fluorescent channel viewing capability as well as a composite image. Each cell image is annotated with ancillary statistical data regarding relative nuclear size, fluorescent intensities, and comparative fluorescent intensities. Each HD-CTC candidate is presented in a field of view with sufficient surrounding WBCs to allow for contextual comparison between cytomorphologic features of the cell in question versus the background WBCs.

The HD-CTC assay was specifically developed with the clinical environment in mind as well as the need for early technology innovation and future automation. All laboratory processes follow strict standard operating procedures that have been optimized, tested, and validated. Data collection and candidate identification has been automated using specific interfaces that both enable the pathologist's decision making and subsequent tracking of these decisions. Specifications for both complete automation and adaption to routine settings will arise from this early research framework.

Cell line experiments

Four aliquots from the donor (2 mL each) were spiked with varying numbers of SKBR3 cells to produce 4 slides with approximately 300, 100, 30, and 10 cancer cells per slide. The 16 slides were then processed and analyzed by a single operator according to the HD-CTC sample preparation protocol. A single instrument was used to image all 16 slides.

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