The ability to isolate and molecularly analyze circulating tumor cells is becoming a reality.

Circulating Tumor Cells: A Window Into Tumor Development and Therapeutic Effectiveness

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Background: Circulating tumor cells (CTCs) are an important diagnostic tool for understanding the metastatic process and the development of cancer.

Methods: This review covers the background, relevance, and potential limitations of CTCs as a measurement of cancer progression and how information derived from CTCs may affect treatment efficacy. It also highlights the difficulties of characterizing these rare cells due to the limited cell surface molecules unique to CTCs and each particular type of cancer.

Results: The analysis of cancer in real time, through the measure of the number of CTCs in a “liquid” biopsy specimen, gives us the ability to monitor the therapeutic efficacy of treatments and possibly the metastatic potential of a tumor.

Conclusions: Through novel and innovative techniques yielding encouraging results, including microfluidic techniques, isolating and molecularly analyzing CTCs are becoming a reality. CTCs hold promise for understanding how tumors work and potentially aiding in their demise.

Introduction

Cancer is a comprehensive term that includes a group of diseases characterized by host cells growing without control. It has a multifactorial origin and, even though many cancers are directly associated with risk factors in modern life, cancer is an ancient disease, with the oldest confirmation of metastatic carcinoma dating to 1200 BC. Cancer is a major health problem worldwide, and 1 of every 4 deaths in the United States is attributed to cancer. The search for biomarkers that allow early detection and may therefore affect the outcome of the disease is critical. The ability of cancer cells to spread in the body, producing metastasis, is one of the most relevant characteristics of the disease and the cause of most cancer deaths. Circulating tumor cells (CTCs) are thought to originate from primary tumors and, due to evolutionary pressures, acquire a genetic heterogeneity that gives them the potential to reach the circulation and colonize distant organs, thus gaining access to better nutrients and biological niches to enhance their survival. Conceptually, this idea is more than 140 years old; in 1874, De Morgan proposed that cells from a primary tumor can escape and travel through surrounding tissue along the lymphatics, or blood vessels, to invade new areas. Ashworth was the first person to observe cells in the peripheral blood that he characterized as “exactly...
in shape, size, and appearance like to those of the tumours,” but the first systematic study on smears of blood from individuals with cancer showing CTCs in 43% of cases was not performed until 1934.7

Therapeutically, CTCs are primarily thought of as a “liquid” biopsy specimen — a snapshot of tumor cells in the circulation at a specific point in time — and their presence has been linked to poor survival rates in metastatic breast,8,12 colon,13-15 and prostate cancers.16-20 A major advantage of using CTCs is that a simple blood draw is faster and less invasive than collecting a tumor sample via a surgical procedure; furthermore, some tumors are in locations that prohibit biopsies. In addition, individual CTCs may contain unique genetic information essential to understanding the tumor biology and the metastatic process of an individual patient’s tumor. This, of course, is a major oversimplification of the heterogeneous nature of tumors, CTCs, and the complexity of the metastatic process, particularly when considering that the general consensus is that CTCs are heterogeneous and can differ between different cancers as well as within an individual patient.21

CTCs have been garnering attention from researchers during the previous decade, and attempts to apply discoveries about individual CTCs to the cancer clinic for individual patients is a major goal of many researchers and health care professionals. The presence of CTCs is one of many predictors of overall survival rates in patients with early stage22 and metastatic breast cancer,8,22-25 and their presence has shown to have a superior overall survival prognostic value compared with serum tumor markers, such as carcinoembryonic antigen and cancer antigen 15-3.26 CTCs provide equivalent or superior prognostic information as radiographic methods but without the possible health risks associated with some of them.27-29

Disseminated tumor cells are a subtype of CTCs localized in bone marrow that may also provide prognostic information in many types of cancer.15,30-32 A retrospective study of metastatic breast cancer showed a significantly higher number of tumor cells in bone marrow compared with the corresponding peripheral blood stem cells,33 but comparative studies have showed variable concordance rates.34 However, bone marrow biopsy is invasive and requires special techniques; in addition, oftentimes patients experience significant discomfort during the procedure. Thus, the study of CTCs is more favorable because CTCs can be obtained through a simple peripheral blood draw.

Currently, the major advantage of liquid biopsy is to measure treatment effectiveness. CTCs have been extended to many types of cancer as biomarkers for malignancies, and this trend is likely to continue. However, because CTCs are sparse (diluted by billions of other cells) and possibly unstable in a typical blood sample, they pose technological challenges for their detection, isolation, and characterization.

Moving beyond the simple isolation and quantification of CTCs in blood samples from patients with cancer is the possibility of analyzing the functional, molecular, and genetic alterations in these cells, and applying this information to improve cancer treatment in “real time.” If realized, this possibility will revolutionize the analysis, monitoring, and treatment of cancer. Can essential information about a tumor be gathered and understood before it seeds and before the heterogeneity morphs it into another form that will lend current therapies ineffective? Many reviews cover the different aspects of CTCs, illustrating the continued interest in this field of study.15,34-42 The current review will briefly describe CTCs, and then focus on developments to further characterize them and the utilization of CTCs as biomarkers for cancer. We will address the potential problems of phenotypically identifying CTCs, isolating them via microfluidic techniques, and using next-generation sequencing to molecularly characterize CTCs.

Relevance

Currently, CTCs are used as a biomarker associated with the status of metastatic cancer. One test alone has been approved by the US Food and Drug Administration for enumerating CTCs in metastatic breast, colorectal, and prostate cancers, and that is the CellSearch System (Janssen Diagnostics, Raritan, New Jersey).53 The test enumerates CTCs and is commonly used before and after a given treatment. If the number of CTCs remains the same or increases, then the treatment may be ineffective; however, if the number of CTCs decreases, then the treatment may be effective. This concept is important because many studies have shown that as few as 3 to 5 CTCs in 7.5 mL of blood can lead to a poor prognosis in terms of progression-free and overall survival rates.10,13,16 CellSearch identifies CTCs as captured circulating cells expressing the cell-surface marker epithelial cell adhesion molecule (EpCAM; CD326), cytokeratin (CK; 8, 18, and 19), and having a positive result on the 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. In addition, CTCs must be negative for the common leukocyte marker CD45, and the morphology (intact cell membrane) and size (> 4 × 4 μm2) of the CTCs must also be taken into account.13,16,28 Studies with the CellSearch System and others have shown that high numbers of CTCs are associated with shorter disease-free and overall survival rates.10,44-53 The presence of 1 or 2 CTCs in patients with nonmalignant disease or in healthy individuals has been detected, but the frequency of appearance in these conditions is rare.47,52,53
Pliability

Most human solid tumors contain an heterogeneous population of cancer cells that originate from epithelial cell types; many of these are shed on a daily basis into the blood, where they survive a couple of hours. Some are apoptotic, whereas others are destroyed by the immune system, even though the immune system of patients with cancer is significantly diminished. But the most relevant patient characteristic is that few cells have the potential to form new metastases, and these cells are sometimes known as cancer stem cells. However, as documented by Plaks et al., more studies are necessary to clarify the relationship between cancer stem cells and CTCs.

Epithelial tumor cells acquire characteristics that allow them to leave the primary tumor in a process known as the epithelial–mesenchymal transition (EMT), which is normally part of embryonic development but, in cancer, is associated with an increase in invasion, metastasis, and drug resistance. The EMT process has been extensively reviewed and will be touched on here to illustrate the heterogeneity of these cells, exemplifying the challenges when identifying and isolating CTCs.

The initiation of EMT is directed by signals in the tumor cell and the surrounding non-neoplastic matrix, but the central characteristic of the EMT is associated with a decrease in members of the cadherin family, particularly E-cadherin expression, which allows tumor cells to detach and move to the surrounding areas, lymph nodes, and enter the blood vessel system as CTCs. The overexpression of N-cadherin has also been observed in prostate cancer and is associated with tumor cell motility and cancer progression in breast cancer. Moreover, evidence reveals heterogeneous cadherin switching depending on the site of origin of the carcinoma. Complex communication between different cellular events control the EMT process, including transforming growth factor \( \beta \) family members associated with the plasticity of epithelial cells, the overexpression of transcription factors, such as snail/slug, zinc finger E-box binding homeobox 2, forhead box C2, goosecoid, twist family basic helix-loop-helix transcription factor 1, and activation of members of the Src tyrosine kinase family. EMT cells in breast cancer show resistance to conventional therapies and surface markers associated with stem cells CD44+/CD24−/low. The surviving CTCs travel through the circulatory system to distant organs where the reciprocal process of the mesenchymal–epithelial transition allows them to invade the tissue.

Some tissues may be predisposed to metastasis to specific cancers, which Paget stated as early as 1889: “In cases of cancer of the breast, it is strange how often the liver is the seat of secondary cancer.” Indeed, the bone, liver, lungs, and brain are all sites for metastatic dominance. Currently, Paget’s “seed and soil” hypothesis is still valid, but metastasis is now recognized as a dynamic process whose outcome depends on the interfaces with the surrounding homeostatic mechanisms that will differentiate the cell from the original tumor. Even with metastasis, which is normally assumed as a late-stage process after the tumor reaches considerable size, evidence exists that invasion could occur in the early stages of disease and then stay clinically dormant.

**Novel Isolation Techniques**

CTCs are difficult to isolate because so few are present in the general circulation; in addition, blood is a complex liquid tissue composed of billions of red corpuscles and hundreds of millions of white blood cells per milliliter, thus presenting technical challenges for the identification and isolation of CTCs. Many common methods for the enrichment of these rare cells have been previously reviewed, and a summary of these select techniques is shown in the Table.

Because many of these techniques have been reviewed elsewhere, we will focus on novel CTC isolation techniques primarily using microfluidics that decrease CTC cell loss, increase recovery rates, use small volumes of blood, and enable the molecular analysis of individual CTCs. Their current advantages and disadvantages are also reviewed.

Two recent studies evaluated the ability to capture and genetically analyze CTCs through gravity-fed microfluidic devices. The first used photolithography and deep-reactive ion etching to create a micronetwork of cell-trapping chambers of 20 × 25 × 30 microns containing 8 to 10 micron pore channels. From this master silicon template, a soft, elastomeric negative mold was forged by pouring and curing against the silicon master. The final microsubstrate was created by hot embossing a plastic plate made of cyclic olefin polymer against the elastomeric negative mold. A thin plastic laminate was then laminated against the cyclic olefin polymer to create the final microchip that allowed blood cells to flow through, leaving the larger CTCs in the chamber. Using the breast cancer cell lines MCF7, MDA-MB-231, and SK-BR-3 spiked into phosphate-buffered solution and the breast cancer cell lines MCF7, MDA-MB-231, and SK-BR-3 spiked into phosphate-buffered solution and human blood, the researchers captured more than 80% of the CTCs. The first used photo lithography and deep-reactive ion etching to create a micronetwork of cell-trapping chambers of 20 × 25 × 30 microns containing 8 to 10 micron pore channels. From this master silicon template, a soft, elastomeric negative mold was forged by pouring and curing against the silicon master. The final microsubstrate was created by hot embossing a plastic plate made of cyclic olefin polymer against the elastomeric negative mold. A thin plastic laminate was then laminated against the cyclic olefin polymer to create the final microchip that allowed blood cells to flow through, leaving the larger CTCs in the chamber. Using the breast cancer cell lines MCF7, MDA-MB-231, and SK-BR-3 spiked into phosphate-buffered solution and human blood, the researchers captured more than 80% of the CTCs. They were also able to show the differential expression of the cancer biomarkers PanCK, ERBB2 (formally known as HER2), E-cadherin, and vimentin in the cell lines tested by immunostaining antibodies and fluorescence microscopy.

The second study was conducted by a group from Japan who developed a novel, rapid CTC isolation device using a 3-dimensional palladium filter. The filter was produced by microfabrication technology and consisted of lithography and electroforming pro-
cesses forming 8 micron pores that allowed normal blood cells and leukocytes to flow through while larger CTCs remained in the pocket. Cell-spiking experiments showed a 90% recovery rate across a range of 50 to 5,000 green fluorescent protein-tagged COLM-5 tumor cells. The filter detected and enumerated CTCs from 19 individuals with metastatic breast cancer, 12 individuals with nonmetastatic breast cancer, and 12 healthy volunteers. The average numbers of CTCs detected were 3.37, 0.23, and 0, respectively, suggesting that the filter may have clinical utility. Using COLM-5 cells, the researchers also showed that further analysis could be performed on the filter using immunohistochemistry for ERBB2, ER, and PGR, and genetic analysis for EGFR/KRAS mutations by polymerase chain reaction (PCR), whole genome amplification, and direct sequencing.

The major advantages of these techniques are the capture of EpCAM-negative CTCs and the capability of enabling single CTC genomic analysis. Two major dis-

Table. — CTC Isolation Methods

<table>
<thead>
<tr>
<th>Isolation Method</th>
<th>Mode of Action</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Examples</th>
</tr>
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<tbody>
<tr>
<td>Cell-density separation</td>
<td>Separates cells based on different densities after centrifugation</td>
<td>Separates cells into discrete layers</td>
<td>CTC size and density not uniform</td>
<td>Ficoll Lymphoprep (Stem Cell Technologies, Vancouver, Canada) OncoQuick (VWR, Radnor, PA)</td>
</tr>
<tr>
<td>Cell size by filtration</td>
<td>Filtration to remove smaller cells in the blood</td>
<td>Simple, 1-step process</td>
<td>Not validated for clinical applications</td>
<td>Polycarbonate membrane ScreenCell (ScreenCell, Westford, MA)</td>
</tr>
<tr>
<td>Microfiltration</td>
<td>Photolithography using membranes separated by a precisely defined gap</td>
<td>Bottom membrane supports captured CTCs, minimizing cell stress</td>
<td>Experimental</td>
<td>3-dimensional</td>
</tr>
<tr>
<td>Immunomagnetic</td>
<td>Antibodies specific to epithelial antigens (eg, CK, PSMA, EpCAM)</td>
<td>Can be performed alone or with other methods (eg, qRT-PCR, FISH, flow cytometry)</td>
<td>Variable rates of false-positive and false-negative staining, depending on antibodies used</td>
<td>MACS RosetteSep (Stem Cell Technologies) MagSweeper (Jeffrey Lab, Stanford, CA) CellSearch (Janssen Diagnostics, Raritan, NJ) AdnaTest (Qiagen, Hannover, Germany)</td>
</tr>
<tr>
<td>Direct analysis</td>
<td>High-throughput assay</td>
<td>Uses whole blood</td>
<td>Not validated for clinical applications</td>
<td>Fiberoptic array screening Micro-Hall sensor</td>
</tr>
<tr>
<td>Leukapheresis</td>
<td>Separates leukocytes by passing blood through external filters</td>
<td>Captures many more CTCs due to the volume of blood</td>
<td>Risky procedure and not likely to be used more than once</td>
<td>Micro-Hall sensor</td>
</tr>
<tr>
<td>High-definition CTCs</td>
<td>All nucleated cells retained and stained with fluorescent antibodies to CK, CD45, and DAPI</td>
<td>Enrichment-free system resulting in high sensitivity and specificity rates</td>
<td>Adds high-definition CTC cytomorphology, allowing multiple analysis parameter characterization</td>
<td>Not validated for clinical applications</td>
</tr>
<tr>
<td>Fluorescence-activated cell-sorting analysis</td>
<td>Flow cytometry–based approach of sorting a mixed population of cells using fluorescent anti-EpCAM antibodies</td>
<td>Allows for semiautomated isolation of single cells or cell pools with less operator intervention and higher throughput</td>
<td>Not validated for clinical applications</td>
<td></td>
</tr>
<tr>
<td>Micromanipulation and laser microdissection</td>
<td>Manual microscopy-based techniques used to identify and isolate antibody-labeled cells using a micromanipulator or micropipette</td>
<td>Can isolate individual cells</td>
<td>Can be harsh on fragile, sticky cells during transfer</td>
<td></td>
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</tbody>
</table>

CK = cytokeratin, CTC = circulating tumor cell, DAPI = 4′,6-diamidino-2-phenylindole, EpCAM = epithelial cell adhesion molecule, FISH = fluorescence in situ hybridization, MACS = magnetic-activated cell sorting, PSMA = prostate-specific membrane antigen, qRT-PCR = quantitative reverse transcription–polymerase chain reaction.
advantages of these methods are that the US Food and Drug Administration has not yet approved them for use and they lack the validation required to be used in a clinical setting. In addition, size filtration–based approaches similar to the 2 reviewed here can have significant red and white blood cell contamination if the pores are plugged with CTCs. This could hinder downstream genetic analysis by increasing the DNA background contamination.

Because EpCAM expression may be lost during EMT, microfluidic devices are being developed to capture CTCs — in particular, EpCAM-negative CTCs — by other means. Galletti et al developed a geometrically enhanced, differential immunocapture microfluidic device using anti-HER2 antibody-coated microposts to isolate CTCs (DAPI+, CK+, CD45−) from patients with breast and gastric cancers. Notably, HER2 expression is correlated with poor prognosis and plays an important role in the metastasis and invasion of breast cancer to surrounding tissues during EMT. The researchers optimized their system using breast cancer cell lines and tested it in individuals both positive and negative for HER2 and found CTCs. This may be an alternative method for evaluating EpCAM-negative CTCs. The same system has also been developed for prostate cancer using a monoclonal, anti-prostate-specific membrane antigen antibody.

A novel, high throughput spiral microfluidic device with a trapezoidal cross-section that relies on the microchannel dimensions and fluidic forces for the size-based separation of CTCs was developed and tested in patients with metastatic breast and lung cancers. The device has a low cost and can process 7.5 mL of peripheral blood in 8 minutes, and its capture efficiency rate is approximately 80% using spiked MCF7, T24, and MDA-MB-231 cell lines. Analysis of patient samples revealed CD24+CD45− and CD24−CD45− cancer stem cell subpopulations and EpCAM+pan-Ck+ cells, which are not currently identified in the traditional EpCAM-positive CTC capture methods. The system is also amenable to fluorescence in situ hybridization and other molecular techniques following CTC isolation.

Sheng et al developed a geometrically enhanced, mixing, high-performance microchip for the efficient capture of pure CTCs. After testing with anti-EpCAM antibody–coated surfaces within the specialized staggered herringbone grooves in the microchip, they found an optimal flow rate of 1 µL/second, a flow velocity of 0.75 mm/second, and a maximum sheer stress of 0.38 dyn/cm. The researchers achieved a capture efficiency rate of 90% of the EpCAM-positive human pancreatic cancer cell line L3.6pl. After spiking between 50 and 50,000 labeled L3.6pl cells into lysed or whole blood, they achieved a 92% capture rate from lysed and a 89% capture rate from whole blood. This method of CTC visual microscop 116 This method of CTC visual microscop ic enumeration was also amenable to isolation via trypsinization and high flow rate washing, recovering more than 60% of the CTCs with a high viability for either CTC culture or further characterization. However, a drawback to this method was that it showed high levels of contaminating leukocytes, especially from patients with pancreatic cancer (> 24,000/mL) compared with healthy donors (~ 3,500/mL).

An improvement of existing technology uses microscale vortices (Vortex Chip [University of California, Los Angeles]) and inertial focusing (which uses fluid inertia through shaped microchannels to align cells at high flow rates) for the high-purity isolation of the relatively larger CTCs from whole blood. By changing the flow rates, channel heights, and length of the Vortex Chip, researchers optimized the system with 19 micron diameter particles. Using 7.5 mL of blood from 8 individuals with breast and lung cancers, they isolated CTCs (23–317 per 7.5 mL) and reduced leukocyte contamination (57%–94% purity). The major advantages of this improved technology over current technologies are reduced processing time (20 minutes), the applicability of the system to other types of cancer, and maintaining the integrity of CTCs for downstream molecular analysis.

Which one of these isolation methods, if any, will be appropriate for health care professionals evaluating patients with cancer remains an open question. What is clear is that the isolation of these rare cells is imperative for the molecular analysis of CTCs and, ultimately, the personalized patient care for all cancer types.

Limitations

The main limitation of the CellSearch System is that the isolation method utilizes EpCAM expression on the cell surface of the tumor, which is expressed in 75% of cancer types alone (and may be lost in undifferentiated tumors or subclones). In renal cell carcinoma subgroups, EpCAM is expressed in chromophobe, papillary, and oncocytoma types but is significantly absent in clear cell carcinomas, whereas others report decreased EpCAM expression in metastases. Similarly, anaplastic thyroid carcinomas lack EpCAM expression, whereas differentiated and poorly differentiated thyroid carcinomas show over-expression. Brain metastasis from breast cancer has been found without the presence of EpCAM-positive CTCs. Similarly, in different human carcinomas, an approximately 10-fold lower rate of EpCAM expression has been found in CTCs than in primary and metastatic tissues. A significant association exists between positive EpCAM expression and survival rate in esophageal cancer, clear-cell renal cell carcinoma, moderately differentiated colon cancer, and thyroid carcinoma; however, EpCAM expres-
tion has also been associated with a worse prognosis in different types of breast cancer,\textsuperscript{150-152} pancreatic cancer,\textsuperscript{153} gallbladder carcinoma,\textsuperscript{154} esophageal squamous cell carcinoma,\textsuperscript{155} and urothelial carcinoma of the bladder.\textsuperscript{156} For epithelial ovarian cancers,\textsuperscript{157} lung cancer,\textsuperscript{129,50} prostate cancer, and well and poorly differentiated colon cancers, no clear correlation with survival rates has been observed (Fig).\textsuperscript{129} These contradictory data highlight the need to discover other phenotypic markers to more precisely identify CTCs. The CellSearch method could use alternative capture approaches beyond antigens to EpCAM, but these methods would be considered experimental approaches and not readily available to patients.

**Molecular Characterization**

Many methods are used to molecularly characterize CTCs, including those that are protein- or nucleic acid-based (eg, reverse transcription–PCR), as well as microarray analysis and sequencing. These methods and the variety of iterations within each general method have been reviewed in detail by Lowes et al.\textsuperscript{138} Thus, this review focuses instead on massively parallel or next-generation sequencing and the practical applications pertaining to patient care and personalized medicine.

The method of molecular characterization is generating interest because of the multiplexing capability and the generation of clonal evolution information that may point to a key player in the progression of cancer.\textsuperscript{139-142} After enumeration and classification, delving into the genetic makeup of CTCs may be important because such data may provide additional information on the mutational status of the tumor. Although the technologies used to isolate individual CTCs are technically demanding, they are beginning to take shape and become available. The technology now exists to look into the genome of individual CTCs, potentially discovering the genetic drivers that allow tumors to progress and evade therapy. Learning more about the genetic makeup of a tumor may allow researchers to discover new and potentially important driver mutations, give biotechnology investigators the information they need to develop novel drugs that target those mutations, and inform health care professionals on how to better treat and cure more patients with cancer.

However, this is a formidable task; simply knowing that a DNA mutation exists does not necessarily mean it is important for tumor development or that it is the only mutation within the tumor. Temporal, spatial, and biological considerations, among many others, may affect the way an individual tumor is developing, but even this view may be too narrow. There are caveats. For example, given that primary and metastatic tumors as well as CTCs are generally heterogeneous, how many individual CTCs should be characterized to achieve a reasonably confident estimate of the makeup of CTCs in the circulation? Which genetic profile — if there are multiple variations as there undoubtedly will be — represents the most meaningful mutational “snapshot” of the tumor? Which driver mutations will give individual CTCs the selective advantage to seed and create new metastases? These and many other questions must first be answered.

In a molecular analysis of comprehensive genomic profiling of CTCs from patients with stage 4 colorectal carcinoma using array comparative genomic hybridization (CGH) and next-generation sequencing, researchers discovered that the primary tumor differs from a liver metastatic tumor, which also differs from CTCs.\textsuperscript{143} When deep sequencing the primary tumor, they determined that mutations thought to be unique to CTCs instead originated from small subpopulations within the primary tumor.\textsuperscript{145}

As proof of concept, Lohr et al\textsuperscript{144} established a standardized process for isolating, qualifying, and retrieving pure CTCs in a study of metastatic prostate cancer; they also generated and quantified independent libraries for whole exome sequencing from magnetically isolated CTCs. This approach looked to find mutations in early tumor evolution (early trunk) or metastatic precursor (metastatic trunk) mutations but did not emphasize private, somatic, single-nucleotide variant mutations from single CTCs. Rather, the researchers used consensus-based variant calling to obtain a combined 19-CTC composite of the CTC population.\textsuperscript{144} The process worked well for individuals with cancer who had more than 5 CTCs per 3.75 mL of blood and generated high-quality libraries for whole exome sequencing.\textsuperscript{144} The researchers were also able to identify 10 early trunk mutations, including TP53,

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**Figure** — High levels of EpCAM expression in tumor cells are controversial. EpCAM expression is associated with a good prognosis in RCC, esophageal cancer, and thyroid cancer, but with a worse prognosis in breast, pancreatic, and gallbladder cancers. EpCAM expression has no clear correlation with prognosis in epithelial ovarian, lung, and prostate cancers. EpCAM = epithelial cell adhesion molecule, RCC = renal cell carcinoma.
found in the primary tumor and metastasis; 90% of these were found in the CTCs analyzed. This finding implies that the cancer came from a single ancestor and then diverged. Lohr et al also found 56 metastatic trunk mutations present in the primary tumor and metastasis, and 73% of these were also found in the CTCs.

Magbanua et al developed a new CTC isolation method based on immunomagnetic enrichment via the CellSearch System and fluorescent-activated cell sorting followed by genomic analysis, including profiled copy number variation (CNV) by CGH and whole genome amplification. CTCs from 9 individuals with castrate-resistant prostate cancer were successfully profiled and had multiple copy number aberrations, including gains in 8q and losses in 8p, as previously reported. The androgen receptor locus had high CNV in 78% of the cases. Similarly, in metastatic breast cancers, CTCs in 102 of 181 individuals were profiled and revealed a wide range in CNV; serial testing of the CTCs was also performed that confirmed reproducibility and indicated genomic changes over time. In addition, an array CGH analysis confirmed that primary tumors and nodal metastasis were highly correlated with each other but less so with CTCs, suggesting that a CTC copy number analysis might provide information about the progression of the tumor in real time. This can be advantageous for health care professionals who may need to change therapeutic interventions based on the genetic signature of the CTCs.

In a study of individuals with colorectal cancer, a group of researchers isolated single CTCs using a micromanipulator and showed by array CGH, mutational profiling, and microsatellite instability analysis that heterogeneity exists between tissue and CTCs and single CTCs. In addition, transcriptional analysis revealed a down regulation of epithelial markers (EpCAM, CK19, and carcinoembryonic antigen), CTC dormancy (reduced Ki67 and c-Myc expression), and an immune escape phenotype (overexpression of CD47, an antiphagocytic signal expressed on cancer cells that signals to macrophages and dendritic cells) compared with the corresponding tumor tissue. This analysis highlighted the survival mechanisms of CTCs and their ability to escape the immune response against tumors, a signature event in the metastatic process.

In patients with lung cancer, reproducible CNV patterns can be found from single CTCs using multiple annealing and looping-based amplification cycles. Study findings suggest that gains and losses of copy numbers at certain chromosome regions during metastasis are selected for and supply the tumor cells with a survival advantage in the circulatory system. The study researchers also found that a small number of individuals with lung adenocarcinoma (N = 4) exhibited nearly identical global CNV patterns (78% of the gain and loss regions were shared). This finding could suggest the possibility that CTC CNV analysis may be a biomarker for lung adenocarcinoma.

It is likely that the genetic analysis of CTCs will be an important area in the development of biomarkers for cancer. However, questions still remain about whether circulating tumor DNA (ctDNA) may be a better or more useful biomarker than CTC analysis. Evidence is accumulating, and analysis of 62 normal and clinically matched samples showed that CTC DNA returned actionable mutations 3 times more often than ctDNA, and ctDNA produced 7 times more false-positive results and 50 times more variation in sensitivity rates than CTC DNA. These are preliminary data; more studies are needed before such a question can be fully answered.

However, it is likely that ctDNA will be a complementary assay to CTCs, providing information on the global tumor load in a patient and the indicators of the genomic conditions of active and dormant cancer cells undergoing apoptosis. In this setting, CTCs more likely represent a real-time glimpse of the biological and functional condition of viable malignant cells and may provide opportunities for a pharmacodynamic evaluation and rapid treatment switching to optimize therapy for patients with cancer.

Conclusions
Circulating tumor cells (CTCs) play a key role in the metastatic process. Ever since the CellSearch System was first approved by the US Food and Drug Administration for the enumeration of these rare cells and was shown to have prognostic value, emphasis has been placed on using these cells to further evaluate tumors. The ability to have a noninvasive, therapeutically relevant diagnostic test has great appeal for health care professionals and patients alike. Discovering the genetic potential of these cells will no doubt lead to a better understanding of the evolution of tumors in patients with cancer.

The future of CTC research is bright. As researchers develop more precise CTC phenotypic markers, such as ERBB2, EGFR, heparanase, and notch1, a signature found in CTCs of metastatic breast tumors that colonize the brain, the ability to further delineate CTC subsets will become a reality. Although the concept of CTCs is not a novel one, this field of study is still in its infancy. With the development of better isolation methods, coupled with improvements in technologies that allow for the genetic characterization of CTCs, new discoveries about how tumors progress, their metastatic potential, and the time-dependent nature of tumor progression will most certainly occur. However, to achieve reliable and comparable results, researchers must standardize the types of isolation methods they use. It is likely that different isolations methods, with unique limitations, could
give different results.

Many more questions still need answers to realize the potential of CTCs. Because CTCs are a heterogeneous population of cells, single-cell genetic analysis will be required, as will searching the profile(s), determining the metastatic potential of the tumor cell, and understanding the copy number variations of specific genes known to be critical in CTCs. In their study of lung cancer, Ni et al.130 illustrated that all CTCs from a given individual, irrespective of cancer subtype, showed reproducible copy number variation patterns similar to the metastatic tumor in the same individual.

An intriguing advantage of the liquid biopsy and CTC analysis is the ability to serially sample and monitor in real time. Until now, health care professionals have seen snapshots of the tumor, instead of the full picture, and the “average” profile. Monitoring the progression of cancer is a significant advantage for health care professionals who may change therapeutic interventions in their patients based on real-time information. If a treatment course has failed (based on the elevated CTC level), then the patient could be switched to another therapy earlier.

In addition, detailing the genetic makeup of CTCs over time may allow personalized medicine to become a reality by addressing specific mutations rather than using chemotherapeutic approaches that do not selectively kill all tumor cells. It is unlikely that an analysis of CTCs will be the answer to all of the biological questions related to tumors, but it is possible that analyzing CTCs may unlock enough information about them to arm health care professionals with the data needed to improve the quality, efficacy, and effectiveness of therapies aimed at eradicating cancer.

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