ARTICLE TITLE: Micrometastatic Cancer Cells in Lymph Nodes, Bone Marrow, and Blood: Clinical Significance and Biologic Implications

CONTINUING MEDICAL EDUCATION ACCREDITATION AND DESIGNATION STATEMENT:
Blackwell Futura Media Services is accredited by the Accreditation Council for Continuing Medical Education to provide continuing medical education (CME) for physicians.
Blackwell Futura Media Services designates this journal-based CME for a maximum of 1 AMA PRA Category 1 Credit™. Physicians should only claim credit commensurate with the extent of their participation in the activity.

CONTINUING NURSING EDUCATION ACCREDITATION AND DESIGNATION STATEMENT:
The American Cancer Society (ACS) is accredited as a provider of continuing nursing education (CNE) by the American Nurses Credentialing Center’s Commission on Accreditation.
Accredited status does not imply endorsement by the ACS or the American Nurses Credentialing Center of any commercial products displayed or discussed in conjunction with an educational activity. The ACS gratefully acknowledges the sponsorship provided by Wiley for hosting these CNE activities.

EDUCATIONAL OBJECTIVES:
After reading the article “Micrometastatic Cancer Cells in Lymph Nodes, Bone Marrow, and Blood: Clinical Significance and Biologic Implications,” the learner should be able to:
1. Summarize the clinical significance of detecting lymph node micrometastases and isolated tumor cells.
2. Summarize the clinical significance of detecting circulating tumor cells in peripheral blood and disseminated tumor cells in bone marrow.

ACTIVITY DISCLOSURES
No commercial support has been accepted related to the development or publication of this activity.

ACS CONTINUING PROFESSIONAL EDUCATION COMMITTEE DISCLOSURES
Editor, Director of Continuing Professional Education, and ACS Director of Medical Content
Ted Gansler, MD, MBA, MPH, has no financial relationships or interests to disclose.
Deputy Editor and ACS Director of Prostate and Colorectal Cancers
Durado Brooks, MD, MPH, has no financial relationships or interests to disclose.
Lead Nurse Planner and Associate Editor
Marcia Grant, RN, PhD, FAAN, has no financial relationships or interests to disclose.
American Academy of Family Physicians representative and Associate Editor
Richard C. Wender, MD, has no financial relationships or interests to disclose.

AUTHOR DISCLOSURES
Dr. Leong has received personal fees from Myriad, Navidea, and Amgen and grants from Neoprobe and Zipline for work performed outside of the current article.
Dr. Tseng has no conflicts of interest to disclose.

SCORING
A score of 70% or better is needed to pass a quiz containing 10 questions (7 correct answers), or 80% or better for 5 questions (4 correct answers).

INSTRUCTIONS ON RECEIVING CME CREDIT
This activity is intended for physicians. For information concerning the applicability and acceptance of CME credit for this activity, please consult your professional licensing board.
This activity is designed to be completed within 1 hour; physicians should claim only those credits that reflect the time actually spent in the activity. To successfully earn credit, participants must complete the activity during the valid credit period, which is up to 2 years from the time of initial publication.

INSTRUCTIONS ON RECEIVING CNE CREDIT
This activity is intended for nurses. For information concerning the applicability and acceptance of CNE credit for this activity, please consult your professional licensing board.
This activity is designed to be completed within 1.5 hours; nurses should claim only those credits that reflect the time actually spent in the activity. To successfully earn credit, participants must complete the activity during the valid credit period, which is up to 2 years from the time of initial publication.

FOLLOW THESE STEPS TO EARN CREDIT
- Log on to acsjournals.com/ce.
- Read the target audience, educational objectives, and activity disclosures.
- Read the activity contents in print or online format.
- Reflect on the activity contents.
- Access the examination, and choose the best answer to each question.
- Complete the required evaluation component of the activity.
- Claim your certificate.

This activity will be available for CME/CNE credit for 1 year following its launch date. At that time, it will be reviewed and potentially updated and extended for an additional 12 months.
All CME/CNE quizzes are offered online FREE OF CHARGE. Please log in at acsjournals.com/ce. New users can register for a FREE account. Registration will allow you to track your past and ongoing activities. After successfully completing each quiz, you may instantly print a certificate, and your online record of completed courses will be updated automatically.
Micrometastatic Cancer Cells in Lymph Nodes, Bone Marrow, and Blood
Clinical Significance and Biologic Implications

Stanley P.L. Leong, MD1*; William W. Tseng, MD2

Cancer metastasis may be regarded as a progressive process from its inception in the primary tumor microenvironment to distant sites by way of the lymphovascular system. Although this type of tumor dissemination often occurs in an orderly fashion via the sentinel lymph node (SLN), acting as a possible gateway to the regional lymph nodes, bone marrow, and peripheral blood and ultimately to distant metastatic sites, this is not a general rule as tumor cells may enter the blood and spread to distant sites, bypassing the SLN. Methods of detecting micrometastatic cancer cells in the SLN, bone marrow, and peripheral blood of patients have been established. Patients with cancer cells in their SLN, bone marrow, or peripheral blood have worse clinical outcomes than patients with no evidence of spread to these compartments. The presence of these cells also has important biologic implications for disease progression and the clinician’s understanding of the process of cancer metastasis. Further characterization of these micrometastatic cancer cells at each stage and site of metastasis is needed to design novel selective therapies for a more “personalized” treatment. CA Cancer J Clin 2014;64:195-206. © 2014 American Cancer Society.

Keyword: cancer metastasis

Introduction
Cancer metastasis may be regarded as a continuum from its inception in the primary tumor microenvironment through its progression to distant sites, by way of the lymphovascular system. For some solid tumors, including cutaneous melanoma and breast cancer, this complex process often occurs in an orderly fashion, beginning with the sentinel lymph node (SLN) as a possible gateway.1-3 However, this working hypothesis is not a general rule as tumor cells may enter the blood and spread to distant sites. It is important to develop methods to track cancer progression by identifying the micrometastatic cancer cells in the different compartments of metastasis, namely the lymph nodes (including the SLNs), bone marrow, and peripheral blood, before disease is detected clinically or radiologically. Cancer cells that have left the primary site and successfully reached these compartments are then in a position to colonize distant sites, which may lead to the death of the patient.

In this review article, an attempt is made to summarize the clinical patterns of cancer metastasis in melanoma, breast cancer, and several other types of solid tumors. Based on these clinical patterns of metastasis, a general concept may be developed that cancer cells from the primary site will spread to the 3 different compartments. Methods of detecting cancer cells in these compartments will be described briefly. The clinical outcomes for patients with detectable micrometastatic cancer cells in their SLNs, bone marrow, and peripheral blood will be summarized. Potential biologic implications will be discussed.

The SLN as a Possible Gateway to Cancer Metastasis
To date, the concept of the SLN has been well validated in patients with melanoma, breast cancer, and other solid tumors. In general, tumor cells at a specific anatomical site will first drain preferentially to corresponding SLNs before reaching other lymph nodes in the same regional lymph node basin.1,4 As a result, close histopathologic examination of the SLN obtained by surgical excision is critical for patients with early disease, as this provides valuable clinical information regarding the status of disease progression.

1Chief of Cutaneous Oncology, Associate Director of the Melanoma Program, Center for Melanoma Research and Treatment, California Pacific Medical Center and Sutter Pacific Medical Foundation, Senior Scientist, California Pacific Medical Center Research Institute, San Francisco, CA; 2Surgical Oncology Fellow, Department of Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX

Corresponding author: Stanley P.L. Leong, MD, Melanoma Program, Center for Melanoma Research and Treatment, California Pacific Medical Center and Sutter Pacific Medical Foundation, Senior Scientist, California Pacific Medical Center Research Institute, 2340 Clay St, 2nd Fl, San Francisco, CA 94115; leongsx@cpmcri.org

DISCLOSURES: Dr. Leong has received personal fees from Myriad, Navidea, and Amgen and grants from Neoprobe and Zipline for work performed outside of the current article.

doi: 10.3322/caac.21217. Available online at cacancerjournal.com
This relatively orderly pattern of early cancer metastasis from the primary tumor site to the SLNs and then to the regional, non-SLNs before spreading to the distant sites (Fig. 1) is consistent with the spectrum theory of cancer proposed by Fisher, which argues that cancer is already a systemic disease at onset.\(^6\) More recently, Morton et al expanded on these biologic concepts with the idea of the SLN as a “incubator” versus a “marker.”\(^7\) The SLN may function as an “incubator” for subsequent metastasis, perhaps through the selection of more aggressive cancer cell clones, the development of immune tolerance, or a combination of these and other mechanisms. Alternatively, the SLN may be viewed as a “marker” of disease when distant metastasis has already occurred, although perhaps at subclinical levels of detection. In theory, surgical resection of SLNs with metastases during the “incubator” phase will be effective in halting disease progression and preventing further distant metastasis. Conversely, if the SLN is simply a “marker” of disease progression, systemic therapy is needed to destroy microscopic disease that is already present at distant sites.

**Detection of Micrometastasis in SLNs and Regional Lymph Nodes and Clinical Significance**

**Melanoma**

Tumor cells can usually be detected within surgically excised SLNs based on simple morphologic appearance after hematoxylin and eosin (H&E) staining on cut sections of tissue. Using H&E staining, false-positive results may be encountered with melanin-containing macrophages or capsular nevus cells.\(^8\)\(^-\)\(^10\) Therefore, immunohistochemistry (IHC) is an important adjunct to H&E staining by definitively identifying tumor cells based on their tumor markers (HMB-45, MART-1 [melanoma antigen recognized by T-cells 1], tyrosinase) that are more specific for melanoma. With IHC, antibodies with specific affinity for these markers result in a colorimetric (eg, brown) “staining” and the easy discrimination of tumor cells from nontumor cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) offers an even more sensitive molecular level of detecting tumor cell ribonucleic acid (RNA) (glycoprotein 100, MART-1, tyrosinase, and MAGE3), even in the absence of visible tumor cells. The routine clinical application of these molecular techniques, however, remains controversial and challenges persist, including the lack of standardization of laboratory methods.\(^11\)

In contrast to clinically palpable or radiologically visible lymph nodes, which are defined as macrometastasis, the presence of tumor cells identified only by conventional histopathology (H&E and/or IHC) are broadly defined as micrometastasis.\(^12\)\(^,\)\(^13\) Further methods for measuring micrometastatic tumor burden in SLNs have been described in melanoma, including: 1) measurement of the maximum diameter in any direction of the largest lesion on a slide (Rotterdam criteria: less than 0.1 mm, 0.1-1.0 mm, and greater than 1.0 mm)\(^14\); 2) aggregate diameter of all tumor foci\(^15\); 3) percentage of the total lymph node area involved with tumor\(^16\); 4) depth of lymph node invasion from the capsule (criteria of Starz et al)\(^17\),\(^18\); and 5) microanatomic location within the lymph node (criteria of Dewar et al).\(^19\) Although the European Organization for Research and Treatment of Cancer recommends use of the Rotterdam criteria, there is no general consensus as to the best method for measuring micrometastatic tumor burden and a combination of methods may be more reliable than a single method used alone.\(^20\) Isolated tumor cells (ITCs), a term originally used in lymph node staging for breast cancer, are a unique subcategory of micrometastasis defined as a single cell or a cluster of cells detected by histopathology and measuring less than 0.2 mm.\(^21\) In the current American Joint Committee on Cancer (AJCC) TNM staging criteria for melanoma, a distinction is made between macrometastasis and micrometastasis; however, the latter is simply defined as a histopathologically identified tumor deposit of any size, including ITCs, and without a “lower limit” for tumor burden.\(^13\) Molecular-detected tumor cells, however, are currently not included.

SLN status in patients with melanoma has been found to be significantly correlated with prognosis and clinical
Micrometastatic Cancer Cells

outcome, with SLN-positive patients faring much worse than those with negative SLNs.\textsuperscript{14,22-32} When a positive SLN is established by histopathology, approximately 10% to 20% of these patients will be found to have more positive regional lymph nodes in the subsequent completion lymph node dissection (CLND).\textsuperscript{33-38} The outcome of these patients with additional positive disease after CLND is much worse than that among patients without additional disease.\textsuperscript{38} Thus, the non-SLN regional lymph nodes in the CLND may be considered as a unique compartment from the SLN, with progression of metastasis from the SLN to the non-SLN compartment. Furthermore, among patients with positive regional lymph nodes, lymph node micrometastatic versus macrometastatic disease appears to be associated with better 5-year overall survival (67% vs 43%; \textit{P} < .001), according to Balch et al.\textsuperscript{39} In this study, micrometastases were broadly defined as clinically and radiologically occult and the vast majority were in fact identified by SLN biopsy alone.

The clinical significance of micrometastatic disease in the SLN was further highlighted by the Multicenter Selective Lymphadenectomy Trial I (MSLT-I), reported by Morton et al.\textsuperscript{40} As the only prospective trial of lymphadenectomy for the treatment of melanoma reported to date, the MSLT-I randomized patients with predominantly intermediate-thickness melanoma (1.2 to 3.5 mm) with clinically negative regional lymph nodes into 2 groups: 1) those treated with wide local excision (WLE) plus observation (no SLN biopsy) and 2) those treated with WLE and SLN biopsy with immediate CLND performed in those patients with positive biopsies. A positive SLN was defined broadly by histopathology (H&E and IHC) alone and without further definition of micrometastatic tumor burden. In the observation group, patients underwent “delayed” (relative to WLE) CLND after the clinical detection of macrometastatic lymph node disease. The investigators found that for patients with nodal disease, the 5-year disease-specific survival was significantly higher in patients treated with SLN biopsy and immediate CLND versus those treated with observation and delayed CLND when lymph node disease became clinically palpable (72% vs 52%; \textit{P} = .004).\textsuperscript{40} This validates the clinical usefulness of the SLN biopsy procedure and suggests that the presence of micrometastatic cancer cells in the SLN indeed warrants removal.

The results of MSLT-I also suggest that there is validity to the biologic concept of the SLN as an “incubator.”\textsuperscript{41} At the time of WLE, both groups (those treated with SLN biopsy/immediate CLND vs those treated with observation/delayed CLND) had similar primary tumor characteristics and would likely have had similar numbers of patients with SLN micrometastases, with the caveat that patients in the observation/delayed CLND group were not detected as their SLNs were not surgically biopsied. With this assumption, when the tumor-bearing “incubator” SLN and regional lymph nodes were immediately removed, disease progression was halted and survival was improved. In contrast, with delayed CLND, the “incubators” were allowed to remain in situ for a longer period of time, resulting in disease progression and worse survival. The authors of MSLT-I also reported that the number of tumor-involved lymph nodes recovered was higher in the group treated with observation/delayed CLND versus the SLN biopsy/immediate CLND group (3.3 lymph nodes vs 1.4 lymph nodes), supporting the concept of orderly progression from the SLN to the regional non-SLNs.\textsuperscript{40} In addition, in the observation group, more patients had the regional lymph node basin versus the distant basin as the site of first recurrence (13% vs 8%), whereas the opposite was true in the group treated with SLN biopsy/immediate CLND (4% vs 11%).\textsuperscript{40} This supports the theory of orderly progression from the SLN and regional lymph nodes to distant sites.

MSLT-II, which is currently in progress, prospectively randomizes patients with positive SLNs to immediate CLND versus observation (and delayed CLND when lymph node disease is clinically or radiologically detected).\textsuperscript{41} The results of this trial will provide further insight into the clinical usefulness of CLND in patients with positive SLNs as well as the biologic role of the SLN and regional lymph nodes (non-SLN compartment).

The microscopic amount of tumor burden within the SLN in general terms appears to impact clinical outcome.\textsuperscript{38,42} The clinical significance of ITCs and molecularly positive SLNs, however, is arguably less clear. Van Akkooi et al reported that the presence of tumor deposits measuring less than 0.1 mm (based on the Rotterdam criteria) were not associated with changes in clinical outcome and even suggested that these patients be regarded as SLN negative.\textsuperscript{43} In contrast, Scheri et al used a higher cutoff of 0.2 mm to define ITCs and observed worse 5-year and 10-year disease-specific and overall survival compared with SLN-negative patients (89% and 80%, respectively, vs 94% and 87%, respectively; \textit{P} = .02).\textsuperscript{27} For molecularly detected SLN-positive disease, Takeuchi et al used RT-PCR techniques for melanoma-associated genes to upstage 30% of patients with histopathology-negative SLNs and found that presence of these markers was associated with a higher risk of recurrence and worse overall survival.\textsuperscript{44} The criteria for SLN positivity in MSLT-II is expanded to include patients with RT-PCR–detected disease and hopefully the results of this trial will help to provide further insight into the clinical significance of molecularly positive SLNs.
Breast Cancer

In patients with breast cancer, the detection of micrometastatic cancer cells in the SLN follows the same principles as in melanoma, with H&E and of course different tumor markers (eg, cytokeratins [CKs]) for IHC and RT-PCR. As a technical point, with histopathology, breast cancer cells within the SLN can generally be detected by frozen section preparation of tissue in contrast to melanoma, which always requires the permanent section of paraffin-embedded tissue. The advantage of frozen section evaluation is that it allows for immediate (eg, intraoperative) assessment of the SLN for tumor positivity; recent studies, however, have suggested that this technique may not be reliable for the detection of ITC deposits.45

The importance of accurately measuring and categorizing tumor burden in the SLN in patients with breast cancer is recognized, including ITCs. In contrast to melanoma, using current AJCC TNM staging for breast cancer, more detailed pathologic N staging is included.21 Specifically, macrometastases are defined as clinically palpable lymph nodes or tumor burden identified by histopathology and measuring greater than 2.0 mm, micrometastases defined as less than 2.0 mm and greater than 0.2 mm, and ITCs are less than 0.2 mm and/or 200 cells. The presence of ITCs alone are categorized as pNO(I+), and the presence of RT-PCR–detected tumor cells is categorized as pNO(mol+).

The American College of Surgeons Oncology Group Z0011 trial was a recent, major prospective study that examined the need for CLND in patients with early breast cancer with clinically nonpalpable but histopathology positive SLNs.46 In this study, SLNs were considered positive simply if identified by H&E staining; patients with solely IHC-identified micrometastases were actually excluded and subcategorization by tumor burden (eg, ITCs) was not done. All patients underwent lumpectomy to negative margins and SLN biopsy. Those with positive SLNs were then randomized to receive CLND or observation, a design that is very similar to that of MSLT-II, which is currently in progress in patients with melanoma. As reported by Giuliano et al, the 5-year disease-free and overall survival rates were very similar between the 2 groups (82% vs 84% and 92% vs 93%, respectively; difference not statistically significant).46,47 The authors concluded that CLND may not be necessary for select patients with a clinically negative but SLN-positive axilla (eg, those with T1/T2 ductal adenocarcinoma). From a biologic standpoint, this suggests that once the positive “incubator” site of disease is removed (by SLN biopsy), disease progression is halted regardless of whether the next stage of spread is surgically addressed (by CLND). Some patients have also received radiation to the axilla, but the role of radiation has not been well defined in this study.

Support for the results of the Z0011 trial was reported by Viehl et al in a separate study with a median follow-up of 97 months.48 In 234 patients with early breast cancer who underwent SLN biopsy alone (without CLND), the authors found that patients with SLN micrometastasis (identified by H&E staining or IHC for pan-cytokeratin) had similar 8-year disease-free and overall survival rates as patients with negative SLNs. No difference was seen with regard to rates of clinical axillary recurrence between the SLN-positive and SLN-negative groups. In fact, in reviewing the published literature, the authors showed that the rates of axillary recurrence were extremely low in patients with SLN micrometastases (including ITCs) once the SLN alone was removed by excisional biopsy. Taken together with the results of the Z0011 trial, these recent findings in patients with breast cancer suggest that for select patients, removal of micrometastases in the SLN may alone be sufficient to clear the axilla. In a recent single-institution study by Dengel et al in patients undergoing breast-conservation therapy who met Z0011 trial eligibility criteria, 84% were spared CLND without axillary recurrence; however, the median follow-up was short (13 months).49 Further study and validation are needed.

Other Cancers

The SLN concept has also been applied to other solid tumors, including colorectal, gastroesophageal, colorectal, lung, head and neck, vulvar, and penile cancers.50-56 The clinical significance of an SLN with micrometastatic cancer cells and the usefulness of SLN biopsy, however, are less well established compared with findings in melanoma and breast cancer. This may be due in part to the fact that for many of these solid tumors, the lymphatic drainage system is more complicated, which makes the identification of the SLN more challenging.3

The clinical purpose of an SLN biopsy can also vary depending on the type of solid tumor. As in patients with melanoma and breast cancer, the goal of SLN biopsy for those with head and neck and gynecologic cancers is similar: to provide prognostic information while minimizing the extent of lymph node dissection.54,56 In contrast, for patients with colorectal and upper gastrointestinal cancers, the goal of SLN biopsy is simply to increase the accuracy of staging of the lymph node basins by ideally limiting detailed examination to only “critical” lymph nodes.50-52 The number of lymph nodes being removed during resection is a significant predictor of survival and therefore the extent of lymphadenectomy will not be altered by SLN biopsy.57,58
Exceptions to the Paradigm of Orderly Progression

It is important to note, however, that approximately 15% to 20% of the time, cancer cells can spread 1) simultaneously via the lymphatic channels to the SLNs and vascular channels to the distant sites or 2) to the systemic sites alone via the vascular system (Fig. 1). In cutaneous melanoma, follow-up of large series of patients with negative SLNs has demonstrated that in the approximately 10% of those who develop recurrence after WLE of the primary site and SLN excisional biopsy, 50% will manifest disease at distant sites. This suggests that there is indeed a subset of patients in whom disease progression “skips” the SLN completely. In patients with breast cancer, Chia et al reported on 10-year outcomes of over 1000 patients with lymph node-negative disease and who did not receive systemic therapy. The authors found that 10% to 25% of these patients had first recurrence at distant sites, with a higher frequency noted in those patients with larger-size tumors. More recent reports using other databases of lymph node-negative, medically untreated patients with breast cancer (Rotterdam, TRANSBIG, and MAINZ) have suggested that up to 20% to 30% will develop distant metastasis by 5 years. In support of these findings, Braun et al found that in patients with early-stage breast cancer, 31% had micrometastatic disease in the bone marrow, a compartment for cancer progression that will be discussed in more detail below. Of note, in this study, patients were not directly compared with those who underwent SLN biopsy to assess their regional lymph node status. Several other solid tumor types, including most soft tissue sarcomas, also appear to “skip” the regional lymph nodes altogether and disseminate to systemic sites via the vascular system. Melanoma that arises in the uvea of the eye, in contrast to cutaneous melanoma, has a unique propensity for liver metastasis but appears to involve lymph nodes infrequently and typically only in the setting of widespread disseminated disease. Molecular studies are clearly needed to further characterize these different mechanisms of disease spread and what governs the pattern of cancer metastasis in the primary tumor and for a given tumor type.

Interestingly, tertiary lymphoid structures (TLS) found within tumors have recently been reported in a subset of patients with non-small cell lung cancer, colorectal cancer, and liposarcoma and melanoma. TLS are organized clusters of immune cells that morphologically resemble immature lymph nodes. As opposed to the negative impact of a tumor-positive SLN, the presence of intratumoral TLS appears to be associated with better clinical outcome. The biologic role of TLS in these tumors and their relevance to the paradigm of orderly progression in cancer metastasis remains to be defined.

Micrometastatic Cancer Cells Beyond the SLN Gateway

Prior to colonizing a distant site, tumor cells can also be found in the bone marrow and peripheral blood. Tumor cells found in the bone marrow are known as disseminated tumor cells (DTCs), whereas those found in the general systemic circulation or peripheral blood are known as circulating tumor cells (CTCs). Peripheral blood flows freely within the bone marrow as well as through intraosseous channels to send hematopoietic cells within the bone marrow back into the systemic circulation. For practical purposes, the circulation between the blood and bone marrow is essentially connected. Whether DTCs and CTCs are the same clonal population of cells, however, is not known. In addition, DTCs in the bone marrow that have extravasated from the circulation may function over time as a reservoir of dormant tumor cells.

Micrometastatic cancer cells in the bone marrow and blood have important biologic implications. These cells have successfully left the primary tumor microenvironment and, in the majority of cases, the SLN. Therefore, it can be argued that this subpopulation of tumor cells has “passed Darwinian selection” with a more aggressive phenotype that has allowed for survival in solitude from other tumor cells and also the evasion of host immune recognition at the SLN. Thus, DTCs and CTCs are in the best position to colonize distant sites and establish a metastatic tumor. Interestingly, in mouse models of breast cancer, colorectal cancer, and melanoma, Kim et al have shown that CTCs may also return and “self-seed” the tumor of origin, in which the selection of a more aggressive phenotype occurs. To our knowledge, this has not yet been demonstrated to occur in humans.

Detection of Tumor Cells in Bone Marrow and Blood

DTCs can be enriched from a bone marrow aspiration specimen using density gradient centrifugation, such as Ficoll-hypaque. This technique physically separates a mixed cell suspension based on differences in size and internal density, with tumor cells being the largest and most internally complex (ie, dense) compared with normal hematopoietic cells, immune cells, and erythrocytes. DTCs are then definitely identified in the bone marrow most commonly by immunocytochemistry, a colorimetric “staining” technique similar to IHC that has been described for assessing the SLN, but in this case is applied to smears of cells obtained during bone marrow aspiration as opposed to a whole-tissue section. For epithelial tumors (breast, colorectal, etc), surface markers such as CKs or mucins (MUC-1) are used to discern DTCs. Analogous to the SLN, multiple investigators have also reported the increased sensitivity of DTC detection using RT-PCR–based techniques; however, there can be
nonspecific false–positive expression of target genes in normal hematopoietic cells, which currently limits the broad application of these molecular techniques.\textsuperscript{74} EPISPOT (EPithelial ImmunoSPOT), which measures secreted tumor-specific proteins, including CKs, after short-term in vitro culture, has also been reported as an alternative functional or viability assay for DTCs.\textsuperscript{75}

In the peripheral blood, the detection of CTCs is more challenging as there is almost a 10-fold lower frequency of these micrometastatic cancer cells in comparison with DTCs in the bone marrow. CTCs are estimated to be as few as one tumor cell per $10^9$ normal blood cells.\textsuperscript{76} The available technologies for CTC detection are constantly evolving and have been extensively reviewed by others.\textsuperscript{77,78}

To summarize, as with DTCs in the bone marrow, CTCs can be grossly separated from normal blood cells using Ficoll density gradient centrifugation. To increase the specificity of detection, CTCs may be further enriched by selection using magnetic beads against cell surface markers. This may entail negative selection (eg, using CD45, an immune cell marker, to select out and exclude these cells from the peripheral blood specimen). Alternatively, positive selection against specific epithelial tumor-associated surface markers such as EpCAM (epithelial cell adhesion molecule) may be used.\textsuperscript{71,76} The CellSearch system (Veridex LLC, South Raritan, NJ) uses ferrous particles coated with antibodies specific for EpCAM. Magnetic separation then isolates EpCAM-positive CTCs, which are then confirmed by fluorescence microscopy after labeling the isolated CTCs with DAPI (a nuclear marker, to exclude erythrocytes), CD45 (CTCs will be negative), and CKs (CTCs will be positive). Several studies (reviewed below) have shown a good correlation between the presence of CTCs in the blood and disease progression among patients with breast, prostate, and colon cancers, resulting in the US Food and Drug Administration approving the CellSearch system.\textsuperscript{79–81} Molecular techniques based on RT-PCR can also be used for CTC detection, with the same limitations of false-positive expression in normal cells as have been described for DTCs. Recent developments include microfluidic devices that take advantage of differences in flow dynamics and/or electrical properties of CTCs compared with normal peripheral blood cells.\textsuperscript{78,82} Combinations of these techniques are brought together in technologies such as the CTC-chip, which isolates tumor cells by precisely controlled microfluidic flow of a sample across an array of microposts coated with EpCAM antibodies.\textsuperscript{78,82}

**Clinical Significance of DTCs in Bone Marrow**

Micrometastatic cancer cells found in the bone marrow (ie, DTCs) at the time of surgical resection or following systemic therapy are associated with recurrence and the subsequent development of metastatic disease at distant sites.\textsuperscript{74,83} The strongest evidence for the clinical significance of DTCs is in breast cancer, although the clinical prognostic value of DTCs has been shown in other solid tumors as well.\textsuperscript{74,83} Braun et al reported their analysis of a pooled database of 4703 patients with primary, nonmetastatic, stage I to stage III breast cancer, which to our knowledge is the largest series to date.\textsuperscript{64} Approximately 31% of all patients had detectable bone marrow micrometastases, which were associated with more aggressive primary tumor characteristics and lymph node-positive disease. The presence of DTCs was associated with the development of metastases at distant sites, as well as worse disease–specific and overall survival, with a follow-up of up to 10 years.\textsuperscript{64} More recently, these findings were confirmed in an updated, pooled analysis of 3 follow-up studies in 676 women with primary operable breast cancer reported by Janni et al.\textsuperscript{84} In both of these studies, DTCs were detected by standard CK-based immunocytochemistry. Using RT-PCR for CK19, MUC-1, and carcinoembryonic antigen RNA to detect micrometastatic cancer cells in the bone marrow, Berois et al analyzed 42 patients with breast cancer and attempted to correlate molecular findings with clinical outcome.\textsuperscript{85} Although CK19 and carcinoembryonic antigen that were detected in the bone marrow using RT–PCR correlated well with positive lymph node status, both assays were also positive in 17% of patients with negative lymph node status. A number of more recent studies have shown the more accurate detection of DTCs using molecular techniques for alternative tumor cell markers and combinations of markers\textsuperscript{74}; however, these techniques are not yet standardized.

DTC detection also has clinical significance in patients with colorectal cancer, although the evidence from available reports to date is not as strong as with breast cancer. Flatmark et al used EpCAM to identify tumor cells in bone marrow aspirates from 235 patients with colorectal cancer.\textsuperscript{86} The authors demonstrated the feasibility of an immunomagnetic rosette separation technique with a 17% detection rate for DTCs. A trend toward a higher frequency of bone marrow positivity was observed from Dukes stage A to stage B to stage C. Further studies using EpCAM and other colorectal cancer cell markers (CK20, CK18) were recently reviewed by Steinert et al.\textsuperscript{87} The authors concluded that the correlation between the presence of DTCs in the bone marrow and various clinical parameters is, at best, mixed.

**Clinical Significance of CTCs in Peripheral Blood**

Similar to DTCs, much of the evidence to support the clinical significance of CTCs comes from patients with breast cancer, particularly those with known metastatic disease.
In these patients, the detection of CTCs in the peripheral blood has significant prognostic value. This was demonstrated by Cristofanilli et al., in which the EpCAM-based CellSearch assay was used to detect and measure CTC burdens in 177 patients with metastatic breast cancer prior to and after the initiation of systemic therapy. At least 2 CTCs were detected in the blood samples from 61% of all study patients with metastatic breast cancer. Using a cutoff of 5 CTCs per 7.5 mL of whole blood, the authors found that patients with a higher tumor burden had worse progression-free and overall survival (2.7 months vs 7 months and 10.1 months vs 18 months, respectively; both P < .001) compared with patients with a lower tumor burden. This result was consistent both before and after treatment. A recent large meta-analysis of 49 CTC studies encompassing 6825 patients verified the prognostic value of CTCs in breast cancer, regardless of the detection method used and time point of blood withdrawal. In patients with metastatic disease, the authors reported a hazards ratio of 1.78 (95% confidence interval [95% CI], 1.52-2.09) for progression-free survival and 2.33 (95% CI, 2.09-2.60) for overall survival. In patients with breast cancer, CTCs may also provide a measure of the effectiveness of systemic chemotherapy. The usefulness of CTC detection for adjusting systemic therapy in patients with metastatic breast cancer was formally evaluated in the Southwest Oncology Group S0500 trial (NCT00382018; clinicaltrials.gov), the results of which have not yet been reported to our knowledge. Several other large, prospective, “interventional” trials examining the usefulness of CTCs in the management of patients with breast cancer are ongoing.

CTCs were also studied using the CellSearch assay in patients with metastatic castration-resistant prostate cancer following chemotherapy, as reported by de Bono et al. Using a cutoff value of 5 CTCs per 7.5 mL of blood, the authors found that among 231 patients, those with higher CTC burdens detected before treatment or at multiple time points after treatment (up to 20 weeks) had a shorter overall survival. CTC counts were found to correlate with better overall survival compared with changes in prostate-specific antigen. Cohen et al., used the CellSearch assay to detect CTCs in 430 patients with metastatic colorectal cancer. Using a lower cutoff value of 3 CTCs per 7.5 mL of blood, the authors similarly observed worse progression-free and overall survival for those patients with a higher versus those with a lower tumor burden during the course of systemic therapy.

Several investigators have used molecular techniques (RT-PCR) to detect CTCs and to determine their clinical significance. Koyanagi et al collected blood specimens from 87 patients with metastatic melanoma before and after induction biochemotherapy and during maintenance. The expression of 5 melanoma-specific CTC markers (MART-1, GalNAc-T, PAX-3, MAGE-A3, and microphthalmia-associated transcription factor [MITF]) was evaluated. The presence of these CTC markers using RT-PCR was strongly correlated with treatment response and clinical outcome. On multivariate analysis, the presence of CTC markers after 2 cycles of biochemotherapy was found to be an independent prognostic factor for disease progression (P < .0001) and overall survival (P = .0005).

While the clinical significance of CTCs was initially established in the metastatic setting as described above, more recently the importance of these micrometastatic cancer cells has also been shown in patients with less advanced disease. In patients with breast cancer, the large recent meta-analysis described above included patients with early disease for whom CTCs were associated with a hazards ratio of 2.86 for disease-free survival (95% CI, 2.19-3.75) and 2.78 for overall survival (95% CI, 2.22-3.48). Hayashi et al also reviewed the literature and found 5 studies in patients with nonmetastatic primary disease in whom CTCs were measured before and/or after systemic therapy. All studies demonstrated statistically significantly worse disease-free and overall survival with the positive identification of CTCs. Interestingly, among these studies, Ignatiadis et al and Xenidis et al used RT-PCR–based techniques to identify CTCs (based on CK19 and other markers), with detection rates of up to 41% compared with 8% to 23% for the CellSearch assay. In patients with colorectal cancer, Peach et al, and Rahbari et al reported literature reviews and meta-analyses of 14 studies and 36 studies, respectively, of CTCs in patients with primary disease. Both studies concluded that the detection of CTCs is associated overall with worse clinical outcomes in terms of recurrence rates and survival. In patients with melanoma, Hoshimoto et al retrospectively examined CTCs using multimarker RT-PCR in the peripheral blood collected from 331 patients enrolled in a multicenter adjuvant vaccine trial. These patients all had stage III disease (lymph node-positive disease) and underwent lymphadenectomy with no evidence of disease prior to enrollment in the trial. The presence of 2 of 3 CTC markers was significantly associated with worse recurrence-free and distant metastasis-free survival.

Advantages and Current Limitations of CTC Detection in the Blood Versus DTC Detection in the Bone Marrow

The potential clinical advantages of testing for CTCs in the blood are manifold. First, CTC detection can provide diagnostic information regarding the stage of cancer progression and, in patients with primary disease or those with no evidence of disease, prognostic information with which to estimate the risk of recurrence. This information
can be obtained prior to the development of clinical or radiologically detectable distant disease. Second, patients with cancer may be stratified by the level of CTCs (eg, 5 CTCs per 7.5 mL of blood) and more objectively monitored for responses to treatment, literally on a “cellular level.” A successful treatment should result in the disappearance of CTCs in the blood: a complete “microscopic” response. Third, the emergence of resistant clones may be identified during treatment and such clones may be targeted with an alternative therapy. An example of this phenomenon is the treatment of patients with human epidermal growth factor receptor 2 (HER2)-positive breast cancer with trastuzumab. Several studies have shown the emergence of HER2-positive CTCs (and DTCs) in patients with breast cancer with HER2-negative primary tumors.98-100 HER2 gene amplification can be acquired during breast cancer progression101; therefore, an additional subset of patients with breast cancer may benefit from treatment with trastuzumab based on HER2 detection in CTCs. Fourth, from a research standpoint, CTCs (and DTCs) offer a unique opportunity to characterize these cells in detail at the subcellular DNA, RNA, and protein levels. Through this type of characterization, novel and more selective therapeutic targets may be identified.74,76

The detection of DTCs in the bone marrow can in theory provide much of the same information described above for CTCs in the blood. For the patient, however, the detection of CTCs in the peripheral blood is preferable, given the relative ease of venipuncture compared with bone marrow aspiration (done through the iliac crest or sternum), which is more invasive and usually more painful. For the investigator, the ease of obtaining peripheral blood also translates into the ability to obtain serial measurements for clinical and/or research purposes.

Significant technical challenges, however, still exist with the detection of CTCs in the blood. As mentioned previously, micrometastatic cancer cells are much more rare in the peripheral blood than in the bone marrow. This issue may be even more exaggerated in patients with primary disease or no evidence of disease compared with those with known metastatic disease who have a higher clinically detectable tumor burden. RT-PCR–based techniques that examine tumor RNA may increase the sensitivity of detection, as shown in the abundance of reports using the molecular identification of CTCs in patients with primary disease. As discussed, however, caution must be used in interpreting these data as false-positive results with normal nontumor cells exist and in general, these molecular techniques are still not standardized.

With CTCs, an important biologic limitation also exists that is emerging based on better understanding of the basic science of the metastatic process. Epithelial-to-mesenchymal transition (EMT) is the concept that epithelial cells develop a more mesenchymal phenotype and genetic program in preparation for leaving the primary tumor microenvironment and metastasizing to distant sites.102 During EMT, a loss of epithelial markers including EpCAM is observed. Thus, CTCs with an EMT phenotype may be missed by current methods of detection, including the CellSearch assay.103 Micrometastatic cancer cells undergoing EMT are arguably the most critical cells; in fact, Mani et al demonstrated in patients with breast cancer that EMT cells resemble cancer-initiating cells or “stem cells” both in terms of surface marker expression and functional properties.104 Therefore, to better and more accurately capture the most clinically relevant CTC subpopulations, more appropriate markers (eg, mesenchymal) need to be developed and tested. Recent reports have begun to explore relevant EMT markers on CTCs in patients with breast and colorectal cancer.105,106

Conclusions
Cancer metastasis may be regarded as a progressive process from its inception and development in the primary tumor microenvironment to its ultimate spread through the lymphovascular system to distant sites. For some solid tumors, including cutaneous melanoma and breast cancer, disease progression occurs in an orderly fashion in general, first through the SLN as a possible gateway for distant metastasis (Fig. 1).7 Subsequently, tumor cells can be identified in the bone marrow (DTCs) and peripheral blood (CTCs). However, this may serve only as a working hypothesis but not as a general rule as tumor cells may enter the blood and spread to distant sites, bypassing the lymph nodes. In this review, we have attempted to summarize the available methods for the detection of micrometastatic cancer cells in each of these 3 compartments, show evidence to support the clinical significance of these cells, and discuss their potential biologic implications whenever possible. We have also tried to place micrometastatic cancer cells within the context of recent major cancer trials, including MSLT-I and the Z0011 trial. These micrometastatic cancer cells are critical as they have effectively left the primary tumor microenvironment and are in a strategic position to colonize distant sites and establish metastatic disease, which is ultimately responsible for the death of the patient. Overall, the importance of micrometastatic disease in solid tumors is increasingly being recognized. In fact, in the current AJCC TNM staging system for breast cancer, there is now the category of cM0(I+) for asymptomatic patients without clinical or radiographic evidence of distant metastases but in whom the presence of microscopic tumors cells in the bone marrow, blood, or nonregional lymph nodes (tumor deposits measuring less than 0.2 mm) is noted.21

Although not discussed in this review, it is equally important to study the fourth compartment, the distant site, in which complex interactions with the local microenvironment...
occur and dictate the fate of these newly arrived tumor cells. This compartment encompasses the events at the “soil” site that allow for implantation of the CTC “seed,” according to the Paget theory of cancer metastasis.\textsuperscript{107} Micrometastatic cancer cells at the distant site may begin to immediately proliferate or, alternatively, these cells may enter a state of dormancy only to emerge at a later time point to cause clinical disease.\textsuperscript{108-110} To our knowledge, there are currently no reliable methods with which to detect micrometastatic cancer cells at the distant site. Recent preclinical studies have also introduced the concept of the “premetastatic niche.”\textsuperscript{111,112} This consists of nonmalignant cells (immune cells, fibroblasts, endothelial cells) recruited to a future metastatic site to “prepare the soil” prior to the actual arrival of tumor cells. Methods to detect this unique microenvironment and the specific nonmalignant cells that make up the premetastatic niche should be investigated for their possible clinical application.\textsuperscript{113}

The challenges are to define each of the compartments in molecular terms and describe how these compartments are interrelated. The major question is whether the cancer cells present at inception within the primary tumor microenvironment are the same as those that move through the lymphatic channels to the SLN/regional lymph nodes or through the vascular channels to the blood/bone marrow and beyond to distant sites. It will also be important to determine which cells within each compartment are actually able to initiate an overt metastasis. Ultimately, these data will enable us to render selective, “personalized” treatment.

References

1. Leong SP. Paradigm of metastasis for melanoma and breast cancer based on the sentinel lymph node experience. J Surg Oncol. 2004;11(suppl 3):192S-197S.

2. Leong SP, Cyd B, Jablons DM, et al. Clinical patterns of metastasis. Cancer Metastasis Rev. 2005;24:221-232.

3. Leong SP, Zuber M, Ferris RL, et al. Impact of nodal status and tumor burden in sentinel lymph nodes on the clinical outcomes of cancer patients. J Surg Oncol. 2011;103:518-530.

4. Boland CM, Gershenson JE. Sentinel lymph node biopsy in melanoma. Cancer J. 2012;18:185-191.

5. Hellman S, Karnofsky Memorial Lecture. Natural history of small breast cancers. J Clin Oncol. 1994;12:2229-2234.

6. Fisher B. Laboratory and clinical research of metastatic disease. Cancer. 1996;20:834-840.

7. Morton DL, Hoon DS, Cochran AJ, et al. “Stealth” melanoma cells in histology-negative sentinel lymph nodes and clinical outcome based on the primary melanoma and the sentinel node. Mod Pathol. 2004;17:747-755.

8. Martinez-Penuela A, Del Olmo J, Boan JF, Idoate MA. Incidental findings in negative sentinel lymph nodes of patients with malignant melanoma: report of three cases. Am J Dermatopathol. 2007;29:104-105.

9. Iakura E, Huang RR, Wen DR, Cochran AJ. “Stealth” melanoma cells in histology-negative sentinel lymph nodes. Am J Surg Pathol. 2011;35:1657-1665.

10. Carson KF, Wen DR, Li PX, et al. Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. J Clin Oncol. 2001;19:3635-3648.
term follow-up. World J Surg. 2005;29:683-691.

33. Reeves ME, Delgado R, Busam KJ, Brady MS, Cotig DG. Prediction of nonsentinel lymph node status in melanoma. Ann Surg Oncol. 2003;10:27-31.

34. Lee JH, Essner R, Torisu-itakura H, Wanek L, Wang H, Morton DL. Factors predicting of tumor-positive nonsentinel lymph nodes after tumor-positive sentinel lymph node dissection for melanoma. J Clin Oncol. 2004;22:3677-3684.

35. Sabel MS, Griffith K, Sondak VK, et al. Predictors of nonsentinel lymph node positivity in patients with a positive sentinel node for melanoma. J Am Coll Surg. 2005;201:37-47.

36. Page AJ, Carlson GW, Delman KA, Murray D, Hestley A, Cohen C. Prediction of nonsentinel lymph node involvement in patients with a positive sentinel lymph node in malignant melanoma. Am Surg. 2007;73:674-678; discussion 678-679.

37. Rossi CR, De Salvo GL, Bonandini E, et al. Factors predictive of nonsentinel lymph node involvement and clinical outcome in melanoma patients with metastatic sentinel lymph node. Ann Surg Oncol. 2008;15:1202-1210.

38. Baehner FL, Li R, Jenkins T, et al. Axillary dissection can be avoided in the majority of clinically node-negative patients undergoing breast-conserving therapy [published online ahead of print August 22, 2013]. Ann Surg Oncol.

39. Wang Z, Dong ZY, Chen QJ, Liu JL. Diagnostic value of sentinel lymph node biopsy in gastric cancer: a meta-analysis. Ann Surg Oncol. 2012;19:1541-1550.

40. van der Pas MH, Meijer S, Hoekstra OS, et al. Sentinel-lymph-node procedure in colon and rectal cancer: a systematic review and meta-analysis. Lancet Oncol. 2011;12:540-550.

41. van der Zaag ES, Bouma WH, Tanis PJ, Ubbink DT, Belman WA, Buskens CJ. Systematic review of sentinel lymph node mapping for colorectal cancer. Ann Surg Oncol. 2012;19:3449-3459.

42. Liptay MJ, D’Amico TA, Nwogu C, et al. Intraoperative sentinel node mapping with technitium-99m in lung cancer: results of CALGB 140203 multicenter phase II trial. J Thorac Oncol. 2009;4:198-202.

43. Robinson K, Holman LL, Moore RG. Update on sentinel lymph node evaluation in gynecologic malignancies. Curr Opin Obstet Gynecol. 2011;23:8-12.

44. Sadeghi R, Gholami H, Zakavi SR, Kakhki VR, Tabasi KT, Horenblas S. Accuracy of sentinel lymph node biopsy for inguinal lymph node staging of penile squamous cell carcinoma: systematic review and meta-analysis of the literature. J Urol. 2012;187:25-31.

45. Kuriakose MA, Trivedi NP. Sentinel node biopsy in head and neck squamous cell carcinoma. J Laryngol Otol. 2009;123:100-110.

46. Dillman RO, Aaron K, Heinemann FS, McClure SE. Identification of 12 or more lymph nodes in resected colon cancer specimens as an indicator of quality performance. Cancer. 2009;115:1840-1848.

47. Smith DD, Schwarz RR, Schwarz RE. Impact of total lymph node count on staging and survival after gastrectomy for gastric cancer: data from a large US-population database. J Clin Oncol. 2005;23:7114-7124.

48. Yee VS, Thompson JF, McKinngn JC, et al. Outcome in 846 cutaneous malignant melanoma patients from a single center after a negative sentinel node biopsy. Ann Surg Oncol. 2009;16:1249-1252.

49. Zogakis TG, Essner R, Wang HJ, et al. Melanoma recurrence patterns after negative sentinel lymphadenectomy. Arch Surg. 2005;140:865-871; discussion 871-872.

50. Chia SK, Speers CH, Bryce CJ, Hayes MM, Olivotto IA. Ten-year outcomes in a population-based cohort of node-negative, lymphatic, and vascular invasion-negative early breast cancers without adjuvant systemic therapies. J Clin Oncol. 2004;22:1630-1637.

51. Schmidt M, Petry IB, Bohn D, et al. Ep-CAM RNA expression predicts metastasis-free survival in three cohorts of untreated node-negative breast cancer. Breast Cancer Res Treat. 2011;125:637-646.

52. Siggelkow W, Boehm D, Gehbard S, et al. Expression of aurora kinase A is associated with metastasis-free survival in node-negative breast cancer patients. BMC Cancer. 2012;12:562.

53. Braun S, Vogl FD, Naume B, et al. A pooled analysis of bone marrow micrometastasis in breast cancer. N Engl J Med. 2005;353:793-802.

54. Leong SP, Nakakura EK, Pollock R, et al. Unique patterns of metastases in common and rare types of malignancy. J Surg Oncol. 2011;103:607-614.

55. Collaborative Ocular Melanoma Study Group. Assessment of metastatic disease status at death in 435 patients with choroidal melanoma in the Collaborative Ocular Melanoma Study (COMS): COMS report no. 15. Arch Ophthalmol. 2001;119:670-676.

56. Dieu-Nosjean MC, Antoine M, Danel C, et al. Long-term survival for patients with non-small-cell lung cancer with intrathoracic lymphoid structures. J Clin Oncol. 2008;26:4410-4417.

57. Coppola D, Nebozhyn M, Khalil F, et al. Unique ectopic lymph node-like structures present in human primary colorectal carcinoma are identified by section gene array profiling. Am J Pathol. 2011;179:37-45.

58. Tseng WW, Demicco EG, Lazar AJ, Lev DC, Pollock RE. Lymphocyte composition and distribution in inflammatory, well-differentiated retroperitoneal liposarcoma: clues to a potential adaptive immune response and therapeutic implications. Am J Surg Pathol. 2012;36:941-944.

59. Messina JL, Fenstermacher DA, Eschrich S, et al. 12-Chemokine gene signature identifies lymph node-like structures in melanoma: potential for patient selection for immunotherapy! Sci Rep. 2012;2:765.

60. Gorges TM, Pantel K. Circulating tumor cells as therapy-related biomarkers in cancer patients. Cancer Immunol Immunother. 2013;62:931-939.

61. Hellman S, Darwin’s clinical relevance. Cancer. 1997;79:2275-2281.

62. Kim MY, Oskarsson T, Acharya S, et al. Tumor self-seeding by circulating cancer cells. Cell. 2009;139:1315-1326.

63. Lin H, Balic M, Zheng S, Datar R, Cote RJ. Disseminating and circulatin tumor cells: role in effective cancer management. Crit Rev Oncol Hematol. 2011;77:1-11.

64. Alix-Panabieres C. EPISPOT assay: detection of viable DTCs/CTCs in solid tumor patients. Recent Results Cancer Res. 2012;195:69-76.

65. Pantel K, Alix-Panabieres C. Circulating tumor cells in cancer patients: challenges and perspectives. Trends Mol Med. 2010;16:398-406.

66. Parkinson DR, Dracopoli N, Petty BG, et al. Considerations in the development of circulating tumor cell technology for clinical use. J Transl Med. 2012;10:138.
206
CA: A Cancer Journal for Clinicians

78. Dong Y, Skelley AM, Merdek KD, et al. Microfluidics and circulating tumor cells. J Mol Diagn. 2013;15:149-157.

79. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351:781-791.

80. de Bono JS, Scher HI, Montgomery RB, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res. 2008;14:6302-6309.

81. Cohen SJ, Punt CJ, Iannotti N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol. 2008;26:3213-3221.

82. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature. 2007;450:1235-1239.

83. Riethdorf S, Wikman H, Pantel K. Review: biological relevance of disseminated tumor cells in cancer patients. Int J Cancer. 2008;123:1991-2006.

84. Janni W, Vogl FD, Wiedswang G, et al. Persistence of disseminated tumor cells in the bone marrow of breast cancer patients predicts increased risk for relapse—a European pooled analysis. Clin Cancer Res. 2011;17:2967-2976.

85. Berrios N, Varangot M, Aizen B, et al. Molecular detection of cancer cells in bone marrow and peripheral blood of patients with operable breast cancer. Comparison of CK19, MUC1 and CEA using RT-PCR. Eur J Cancer. 2000;36:717-723.

86. Flatmark K, Borgen E, Nesland JM, et al. Disseminated tumour cells as a prognostic biomarker in colorectal cancer. Br J Cancer. 2011;104:1434-1439.

87. Steinert G, Scholch S, Koch M, Weitz J. Biology and significance of circulating and disseminated tumour cells in colorectal cancer. Langenbecks Arch Surg. 2012;397:535-542.

88. Zhang L, Riethdorf S, Wu G, et al. Meta-analysis of the prognostic value of circulating tumor cells in breast cancer. Clin Cancer Res. 2012;18:5701-5710.

89. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol. 2005;23:1420-1430.

90. Bidard FC, Fehm T, Ignatiadis M, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer Metastasis Rev. 2013;32:179-188.

91. Koyanagi K, O’Day SJ, Boasberg P, et al. Serial analysis of circulating tumour cells predicts outcome of induction chemotherapy plus maintenance for metastatic melanoma. Clin Cancer Res. 2010;16:2402-2408.

92. Hayashi N, Yamauchi H. Role of circulating tumor cells and disseminated tumor cells in primary breast cancer. Breast Cancer Res. 2012;19:110-117.

93. Ignatiadis M, Kallergi G, Ntoulia M, et al. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer. Clin Cancer Res. 2008;14:2593-2600.

94. Xenidis N, Ignatiadis M, Apostolaki S, et al. Cytokeratin-19 mRNA-positive circulating tumor cells after adjuvant chemotherapy in patients with early breast cancer. J Clin Oncol. 2009;27:2177-2184.

95. Peach G, Kim C, Zacharakis E, et al. Prognostic significance of circulating cells following surgical resection of colorectal cancers: a systematic review. Br J Cancer. 2010;102:1327-1334.

96. Rahbari NN, Aigner M, Thorlund K, et al. Disseminated tumour cells as a prognostic biomarker in colorectal cancer. Br J Cancer. 2011;27:1714-1726.

97. Hoshimoto S, Faries MB, Morton DL, et al. Assessment of prognostic circulating tumor cells in a phase III trial of adjuvant immunotherapy after complete resection of stage IV melanoma. Ann Surg. 2012;255:357-362.

98. Riethdorf S, Muller V, Zhang L, et al. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res. 2010;16:2634-2645.

99. Wulfing P, Borchart J, Buenger H, et al. HER2-positive circulating tumor cells indicate poor clinical outcome in stage I to III breast cancer patients. Clin Cancer Res. 2006;12:1715-1720.

100. Solomayer EF, Becker S, Pergola-Becker G, et al. Comparison of HER2 status between primary tumor and disseminated tumor cells in primary breast cancer patients. Breast Cancer Res Treat. 2006;98:179-184.

101. Meng S, Tripathy D, Shete S, et al. HER-2 gene amplification can be acquired as breast cancer progresses. Proc Natl Acad Sci U S A. 2004;101:9393-9398.

102. Kang Y, Pantel K. Tumor cell dissemination: emerging biological insights from animal models and cancer patients. Cancer Cell. 2013;23:573-581.

103. Joosse SA, Pantel K. Biologic challenges in the detection of circulating tumor cells. Cancer Res. 2013;73:8-11.

104. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133:704-715.

105. Yokobori T, Inuma H, Shimamura T, et al. Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis. Cancer Res. 2013;73:2059-2069.

106. Yu M, Bardia A, Wittner BS, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science. 2013;339:580-584.

107. Amerasekera S, Turner M, Purushotham AD. Paget’s “seed and soil” hypothesis revisited. J BUON. 2004;9:465-467.

108. Goss PE, Chambers AF. Does tumour dormancy offer a therapeutic target? Nat Rev Cancer. 2010;10:871-877.

109. Uhr JW, Pantel K. Controversies in clinical cancer dormancy. Proc Natl Acad Sci U S A. 2011;108:12396-12400.

110. Tseng WW, Fukaki N, Leong SP. Metastatic tumor dormancy in cutaneous melanoma: does surgery induce escape? Cancers (Basel). 2011;3:730-746.

111. Kaplan RN, Ribas A, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature. 2005;438:820-827.

112. Peinado H, Lavotshkin S, Lyden D. The pre-metastatic niche revisited. J BUON. 2010;15:871-877.

113. Zoccoli A, Iuliani M, Pantano F, et al. Premetastatic niche: ready for new thera-peutic interventions? Expert Opin Ther Targets. 2012;16(suppl 2):S119-S129.