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Let Me Do More Than Count the Ways: What Circulating Tumor Cells Can Tell Us about the Biology of Cancer

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Abstract: Tumor cells in the circulation of patients with advanced cancers have been described for over a century, but only recently have methods become available to reproducibly and robustly detect these cells in patients with cancer. A variety of methods have been developed to study this phenomenon, reflecting a broad interest in the field. The presence of circulating tumor cells (CTCs) in the peripheral blood of patients with metastatic cancer has been found to be of prognostic significance, and changes in CTC numbers over time appear to reflect treatment outcome. The ability to detect and study CTCs suggests that CTC concentration in blood may be able to be used as an intermediate biomarker in therapeutic trials of novel therapies in cancer patients and that molecular changes in patients' tumors may be able to be detected and addressed with appropriate therapeutic interventions. Studies in patients with early, nonmetastatic cancers are beginning, and some studies indicate that circulating tumor cells can predict outcome in this setting. While the ability to count and characterize circulating tumor cells holds much potential for the future, improvements in and standardization of assay methods need to be made before the potential of this technology is fully realized.

Keywords: Circulating tumor cells; disseminated tumor cells; prognostic factors; predictive factors

Introduction

Circulating tumor cells (CTCs) were first described in 1869 by Ashworth,[1] and until recently could be detected only in patients with heavy tumor burdens in the setting of advanced cancer. Further study of this phenomenon was initially hindered by limitations in cell separation, concentration, and cytologic techniques. In the latter part of the 20th century, the development and clinical application of immunomagnetic separation techniques, immunocytocytology, flow cytometry, fluorescence *in situ* hybridization (FISH), and the polymerase chain reaction laid the groundwork for studies demonstrating that low concentrations of circulating tumor cells could be detected in patients with malignancy. With the ability to detect these cells have come studies investigating the clinical implications of this phenomenon and the characterization of these cells.

How are Circulating Tumor Cells Detected and Enumerated? Because circulating tumor cells are rare, accounting for 1 or fewer cells in 10^5-10^6 peripheral blood mononuclear cells,[2] many assays involve an initial enrichment step to produce a preparation enriched for circulating tumor cells. A number of methods have been described for treating peripheral blood to produce such an enriched product. An incomplete listing of these positive selection methods includes density gradient centrifugation, immunomagnetic separation, and immunoselection under low-flow conditions. An alternative is to use a negative selection technique, in which contaminating peripheral blood mononuclear cells are

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selected for and discarded.[3] Whether or not an enriched product is produced, the number of circulating tumor cells must be enumerated. Methods to accomplish this quantitation include flow cytometric techniques, direct cytometric analysis by a cytologist or an image analyzer, indirect nucleic acid techniques, and microfluidics with immunocapture. The FDA-approved and commercially available CellSearch assay involves immunomagnetic enrichment by positive selection for circulating tumor cells with epithelial cell-specific Ep-CAM antibodies, followed by image analysis.[4] Circulating tumor cells are defined as intact cells which are nucleated, defined as being DAPI positive, and which are characterized immunocytochemically as being (1) positive for staining with a mixture of two phycoerythrin-conjugated antibodies that bind to cytokeratins 8, 18, and 19 and (2) negative for the leukocyte marker CD45. Indirect nucleic acid techniques have been described, using reverse-transcription and the polymerase chain reaction (RT-PCR) to amplify the signal for an epithelial marker which is not normally found in peripheral blood and comparing the number of PCR cycles to cross a required threshold to a standardized curve. This method has been successfully used to quantitate the number of cytokeratin-19 mRNA positive circulating tumor cells in patients with both early and advanced breast cancer.[5,6] A novel assay using a microfluidic device containing microposts coated with antibodies against epithelial cells has been described and studied in a variety of tumor types.[7,8] Short-term culture of circulating cells and identification of secreted proteins has been used to detect circulating tumor cells in an enzyme-linked immunospot (ELISPOT) assay,[9,10] and circulating naked DNA has been used to impute the presence of circulating tumor cells.[11] Microfiltration has been used to separate circulating tumor cells from peripheral blood, and a novel parylene filter has been developed. This system was able to recover approximately 90% of spiked tumor cells in peripheral blood in a 10 min process, and methods to lyse cells on the filter and perform PCR reaction on the lysate have been described.[12]

Are They Tumor Cells? While the methods described above represent technically interesting approaches to the detection of rare events, an important question to address is whether the cells detected by these techniques are, in fact, tumor cells. This is particularly important because many of the methods used to concentrate or identify tumor cells use epithelial markers which can be found on benign as well as malignant cells. The evidence to support the assertion that the cells detected by these methods represent tumor cells include (1) an association between detectable CTCs and the ability to culture tumor colonies from blood or CTC-enriched product, (2) genetic abnormalities in CTCs that are similar to those of intact tumors, and (3) an association between CTCs and the clinical status of the patient.

Tumor colonies have been cultured from mononuclear cell preparations derived from marrow and peripheral blood, and the ability to grow tumor colonies has been found to be roughly associated with the presence of immunocytochemically detectable circulating tumor cells.[2] Fluorescence *in situ* hybridization with centromeric ("chromosome enumerator") probes has been performed on circulating tumor cells and on primary tumors from the same patients.[13,14] In a study of patients with breast, kidney, prostate, and colon cancer, aneusomy was detectable in the CTCs of 25 of 31 patients with detectable CTCs, and the changes in chromosome number were the same in the CTCs and the primary tumor

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in 10 of 13 cases where such a comparison could be made.[13] Using the "CTC chip" technology, mutations in the epidermal growth factor receptor known to occur in nonsmall lung cancers have been found in the circulating tumor cells of lung cancer patients.[8] In some cases, the same abnormalities have been found in the primary tumor and in the CTCs, and in some cases the CTCs have shown evidence of the acquisition of additional abnormalities, consistent with tumor progression. Circulating tumor cells can be detected in the blood of patients with a variety of cancers, but are rare in the blood of patients who do not have known malignancies.[4] Moreover, the number of circulating tumor cells correlates with the stage and aggressiveness of patients' malignancies,[4,7,8] a subject expanded upon below.

Are CTCs Relevant to the Clinical Course of Disease? The number of detectable circulating tumor cells has been found to correlate with clinical outcome in a number of studies.[4,7,8] Furthermore, serial studies have indicated that CTCs can be used to monitor the course of a patient's disease.[7,8,15−17] In a multicenter trial prospectively designed to determine the relationship between CTC number and clinical outcome in patients with metastatic breast cancer, the median progression-free survival of patients with baseline values of 5 or more CTCs per 7.5 mL of blood was 2.7 months, as compared with a progression-free survival of 7.0 months in patients with fewer than 5 CTCs per 7.5 mL of blood.[15] In the same study, the median overall survival was 10.1 months for patients with 5 or more CTCs per 7.5 mL of blood at baseline, as compared to a median survival of >18 months for patients with 0−4 CTCs per 7.5 mL of blood. Importantly, patients whose CTC numbers declined to <5 per 7.5 mL of blood with therapy assumed the more favorable prognosis of those with low CTCs at baseline, and patients whose CTC counts rose to ≥5/7.5 mL at any time tended to show clinical progression soon thereafter, suggesting that the assay could be used to follow the clinical course and response to therapy of patients with metastatic breast cancer.[15,18] Preliminary reports of a confirmatory study have shown similar results.[19,20] Similar findings have now been reported in multicenter prospective trials performed in patients with metastatic colorectal[17] and hormone-refractory metastatic prostate cancer.[16] The ability of early changes in circulating tumor cell numbers to predict the outcome following the institution of a new therapy for metastatic breast cancer has been compared to that of traditional radiologic techniques.[21] In this study, the results of the CTC assay, ascertained at a median of 4.6 weeks, was at least as good as the results of radiologic evaluation at a median of 11.9 weeks in predicting the outcome of patients beginning a new therapeutic regimen. In addition to being available at an earlier point in time, the CTC assay was more reproducible than radiologic interpretation and appeared to add information when the radiographic response was known. More recently, this CTC assay was also found to be superior to serial FDG-PET imaging of patients with metastatic breast cancer.[22]

Pitfalls and Potential: Implications for the Future. The evidence that early changes in CTC number are associated with treatment outcome raises the possibility that changes in CTC number could be used as an intermediate end point in clinical trials in breast cancer. What has not been demonstrated for CTCs (or any other means of evaluating response) is whether making a change in therapy based on the assay result affects therapeutic outcome. The hypothesis that an improved outcome can result from a strategy of changing or continuing therapy based on the results of a CTC assay is being prospectively studied by the US Breast Intergroup in S0500, a clinical trial being led by the Southwest Oncology Group. In this trial, patients with elevated CTCs at baseline and first follow-up are being randomized to switch to an alternative therapy or to continue therapy without change until there is evidence of disease progression on radiographic evaluation or physical examination.

Exploratory studies have demonstrated that molecular changes in circulating tumor cells can be detected. These assays can be done much more easily than can repeat tumor biopsies, and suggest new research and clinical practice paradigms. Evidence of the acquisition of mutations in the EGFR associated with resistance to tyrosine-kinase inhibitors have been described,[7] suggesting that tumors could be

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monitored serially. This would allow, in the investigative setting, mechanisms of resistance to be defined and, in the practice setting, appropriate therapeutic changes to be made on the basis of assays done on circulating tumor cells. The expression of HER2 on circulating tumor cells has been determined by flow cytometry[23] and the presence of HER2 gene amplification by fluorescence *in situ* hybridization.[24] Of interest, FISH assays in CTCs suggested the acquisition of HER2 amplification in circulating tumor cells from primary tumors that were not HER2 amplified.[24]

While the ability to assess the disease state of patients with advanced malignancy promises to improve our understanding of disease and to provide information that is clinically useful, of even greater potential would be the ability to monitor occult metastatic disease in patients with early stage malignancies. At present, the inability to monitor the response to therapy of individual patients with operable cancer has meant that the only means of documenting the effectiveness of therapy in this setting is through the use of large randomized trials with prolonged follow-up. This fact has limited the number of therapeutic questions that can be asked, increased the cost of posing those questions, and resulted in a delay in applying the results as prolonged follow-up is necessary before outcome can be determined and trial results interpreted. An ability to monitor patients in this setting could address some of these problems. Studies are at an early stage, and, depending on the method used, circulating tumor cells can be variably detected in patients with early, operable malignancies. In operable breast cancer, for instance, detectable circulating tumor cells have been reported in 13.9−40.8% of patients using RT-PCR based techniques for mammoglobin and cytokeratin-19, respectively.[6] Using immunocytologic identification, CTCs have been found in 10% of patients,[25] but a method utilizing laser scanning cytometry of antiepithelial cell adhesion molecule-stained epithelial cells from whole unseparated blood, without an enrichment step, reported detectable circulating tumor cells in 90% of patients with operable breast cancer.[26] The largest study of CTCs in early breast cancer was done as part of the multicenter German SUCCESS adjuvant chemotherapy trial. Among 1500 patients with operable node-positive or high-risk node-negative breast cancer, 10% were found to have 1 or more detectable circulating tumor cells in 23 mL of peripheral blood using the CellSearch system; similar numbers were found to have detectable cells following adjuvant chemotherapy. Having detectable CTCs following chemotherapy was found to be associated with an increased risk of relapse and death.[25] This study shows both the problems and potential of this assay. The inability to detect circulating tumor cells in most patients with early cancers may limit the technique, and it must be recognized that the relapse rate in the SUCCESS trial has been relatively low in both CTC-positive and CTC-negative patients. An important step will be for CTC results to predict the final outcome of a randomized trial in which the component arms produce different results; it will be of interest to see whether this proves to be the case in the SUCCESS trial.

The studies cited above highlight an area of concern for the existing technologies. The proportion of patients in whom circulating tumor cells can be detected varies greatly and the numbers of cells reported to be detectable in patients varies widely between the various methods and investigative groups. The question has to be raised as to whether these methods are detecting the same cells and whether the clinical and biologic implications of finding CTCs with a given technique can be generalized. As the methods used become standardized, comparative studies will need to be performed. These problems reflect the fact that this is an area of investigation that remains in its infancy, but is a field with great potential to improve our understanding of malignancy and the metastatic process.

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