

## Circulating Tumor Cells—New Challenges Ahead

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The presence of circulating tumor cells (CTCs)<sup>2</sup> was first described by Thomas Ashworth in 1869 (1). In 1889, Stephen Paget proposed in the first issue of *The Lancet* the “seed and soil” hypothesis, according to which “metastasis depends on cross talk between selected cancer cells and specific organ microenvironment,” a hypothesis revisited many years later by Isaiah Fidler (2). It took more than a century to recognize the critical role that CTCs play in the metastatic spread of carcinomas, that the detection of CTCs is associated with prognosis for many cancers (such as those of the breast, colon, and prostate), and that their enumeration is useful in follow-up (3). On the basis of these developments, the evaluation of CTCs now represents a promising new diagnostic tool, especially for advanced-stage cancer patients, for whom CTCs can be used as a “liquid biopsy” that allows physicians to follow cancer changes over time and tailor treatment accordingly (3).

Given that CTCs are very rare, the very limited amount of available biological sample presents a unique analytical and technical challenge. CTCs can now be isolated, detected, and enumerated with a plethora of highly sensitive analytical methods, such as quantitative reverse-transcription PCR, image-based approaches, and techniques that use microfilter and microchip devices (4). In most cases, the isolation and detection of CTCs depend primarily on their epithelial characteristics, and antibodies to epithelial cell adhesion molecule (EpCAM) and cytokeratins (CKs), respectively, are used for this purpose. At present, the CellSearch<sup>®</sup> system (Veridex) is the only technology that has been cleared by the US Food and Drug Administration for the detection and enumeration of CTCs in patients with metastatic breast, prostate, or colorectal cancer and has been validated by a rigorous clinical-testing program (5). This system is based on a combination of immunocytochemistry and immunofluores-

cence techniques, in which the immunomagnetic selection of CTCs with antibodies specific for the epithelial marker EpCAM is followed by staining of EpCAM-positive cells for CKs (CK-8, CK-18, and CK-19). These cells are subsequently stained with the nuclear stain 4′6-diamidino-2-phenylindole (DAPI) to determine cell viability and for CD45 to exclude leukocytes. The EPISPOT (epithelial immunospot) assay can detect tumor-specific proteins, such as full-length CK-19 released by CTCs (6). The specificity of these assays derives from the fact that blood cells usually lack detectable expression of epithelial markers owing to their mesenchymal origin. The specificity of the CellSearch assay was clearly demonstrated in blood analyses of healthy control individuals and women with benign breast diseases (including fibrocystic disease, fibroadenoma, ductal hyperplasia, and microcalcifications) or other nonmalignant diseases (including diabetes, arthritis, asthma, thyroid abnormalities, and hypercholesterolemia). Fewer than 7.5% of samples contained 1 CTC per 7.5 mL of blood (7), thus showing that patients with benign breast diseases rarely have CTCs in their blood, if at all; however, no similar data are available for age-matched controls with benign colorectal diseases.

This issue of *Clinical Chemistry* includes a report from a leading group of investigators in this field, who have asked the critical question of whether trafficking of normal epithelial cells might occur in the blood circulation of patients with benign colorectal diseases under certain circumstances (8). In this study, Pantel et al. enrolled patients with benign colon diseases, such as diverticulosis, benign polyps, Crohn disease, ulcerative rectocolitis, and colonic endometriosis, and analyzed peripheral blood samples with both the CellSearch system and the EPISPOT assay. They found that in patients with benign colon diseases, positive events that met the criteria for “tumor cells” were detected with both the CellSearch system and the CK-19 EPISPOT assay, whereas none of these events were found in healthy volunteers. Positive events were most frequently detected in patients with diverticulosis and Crohn disease. All positive events lacked expression of CD45, the common leukocyte antigen. These results indicate that certain patients with benign inflammatory colon diseases can harbor viable circulating epithelial cells detectable with current established and validated CTC assays. This finding can be explained by the

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<sup>2</sup> Nonstandard abbreviations: CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; CK, cytokeratin; DAPI, 4′6-diamidino-2-phenylindole; EPISPOT, epithelial immunospot (assay).

fact that epithelial cells from nonmalignant colonic epithelium may enter the bloodstream under certain conditions, such as inflammation, and is consistent with the fact that inflammatory cytokines can stimulate the migration of epithelial cells. Before this study, only a single reverse-transcription PCR–based study had suggested that cells from nonmalignant colonic epithelium might also gain entry to the bloodstream of patients with benign colorectal diseases (9).

According to the findings of Pantel et al. as presented in this report, the potential background of non-malignant epithelial cells in the blood, especially in patients with inflammatory bowel disease, may be an important confounding factor in cancer patients with very low “CTC” counts that could lead to false-positive findings in CTC diagnostics unless strict morphologic criteria or molecular characterization of these cells can be applied. This important finding has to be verified by other CTC methodologies in a larger number of clinical samples.

Another important question raised in this study is the lack of concordance between CTC assays, because only 1 sample was positive with both of the CTC assays used. That finding may be because these methods are based on different capture and detection technologies. Although most CTC-isolation and -detection methods are highly specific and sensitive, few studies have been designed specifically to compare the efficacies of these methods with the same clinical samples. Agreement on a standardized isolation and detection protocol for CTCs is absolutely necessary (4). Cross-validation of findings between laboratories and a universal internal and external QC system for both detecting and enumerating CTCs are urgently needed before these methods can be adopted for routine clinical use. This issue is important, because differences between CTC methods in analytical sensitivity and specificity play a critical role in their utility in the clinical laboratory.

Finally, the authors have raised a very provocative hypothesis: Is it possible that the “false-positive events” detected in this study might be malignant tumor cells at very early stages of cancer development that are already present in some of the benign lesions or the adjacent colon? Could these “false-positive events” therefore represent cancer cells not detected by endoscopic visual inspection and/or routine histopathologic analysis of the resected samples? This possibility cannot be excluded, because tumor cell dissemination appears to occur early after malignant transformation of the primary tumor (10). If malignant CTCs appear at very early stages of carcinoma development, then their detection may have the potential for early diagnosis of colon cancer.

To address this provocative hypothesis, other independent large clinical trials and epidemiologic stud-

ies with long-term follow-up should confirm these results. Before performing such studies, however, the nature of these circulating EpCAM-positive and CK-positive cells has to be elucidated. Molecular characterization of CTCs can provide valuable information on (a) the expression of cancer-specific genes in these cells (11–13), (b) mutations in oncogenes or tumor suppressor genes (14), (c) epigenetic silencing of tumor suppressor genes and metastasis suppressors (15), and (d) fluorescence in situ hybridization–based detection of numerical chromosomal aberrations. As the authors have indicated, false-positive findings may be encountered with the current CTC assays without further molecular analysis of these cells.

In conclusion, the findings of this study have important implications for the use of CTC testing in the clinical laboratory and point to the need for molecular characterization of these cells. Molecular characterization of CTCs is absolutely necessary; simple enumeration will not suffice. The future use of CTCs lies in the molecular characterization of these cells. CTC technologies that are complementary, such as imaging and molecular-characterization methods, should be used in combination to provide a complete view of the malignant nature of these cells. Moreover, standardization of protocols for isolating and detecting CTCs, cross-validation of findings between laboratories, and universal internal and external QC systems for CTC detection and enumeration are necessary and needed.

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