Analysis of Circulating Tumor Cells in Patients with Non-small Cell Lung Cancer Using Epithelial Marker-Dependent and -Independent Approaches

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Introduction: Epithelial circulating tumor cells (CTCs) are detectable in patients with non-small cell lung cancer (NSCLC). However, epithelial to mesenchymal transition, a widely reported prerequisite for metastasis, may lead to an underestimation of CTC number. We compared directly an epithelial marker-dependent (CellSearch) and a marker-independent (isolation by size of epithelial tumor cells [ISET]) technology platform for the ability to identify CTCs. Molecular characteristics of CTCs were also explored.

Methods: Paired peripheral blood samples were collected from 40 chemonäive, stages IIIA to IV NSCLC patients. CTCs were enumerated by Epithelial Cell Adhesion Molecule-based immunomagnetic capture (CellSearch, Veridex) and by filtration (ISET, RareCell Diagnostics). CTCs isolated by filtration were assessed by immunohistochemistry for epithelial marker expression (cytokeratins, Epithelial Cell Adhesion Molecule, epidermal growth factor receptor) and for proliferation status (Ki67).

Results: CTCs were detected using ISET in 32 of 40 (80%) patients compared with 9 of 40 (23%) patients using CellSearch. A subpopulation of CTCs isolated by ISET did not express epithelial markers. Circulating tumor microemboli (CTM, clusters of ≥3 CTCs) were observed in 43% patients using ISET but were undetectable by CellSearch. Up to 62% of single CTCs were positive for the proliferation marker Ki67, whereas cells within CTM were nonproliferative.

Conclusions: Both technology platforms detected NSCLC CTCs. ISET detected higher numbers of CTCs including epithelial marker negative tumor cells. ISET also isolated CTM and permitted molecular characterization. Combined with our previous CellSearch data confirming CTC number as an independent prognostic biomarker for NSCLC, we propose that this complementary dual technology approach to CTC analysis allows more complete exploration of CTCs in patients with NSCLC.

Key Words: Circulating tumor cells, Non-small cell lung cancer, CellSearch, ISET.

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The detection and characterization of circulating tumor cells (CTCs) in cancer patients offer as yet untapped potential to further understand the biology of human cancer metastasis and to identify novel treatment strategies. Recent advances in technology have paved the way to reproducible CTC detection and enumeration and begun to reveal their potential as a real-time, minimally invasive, "virtual biopsy" (reviewed in Hou et al.[1]). The robust, semiautomated CellSearch platform (Veridex, LLC, Raritan, NJ) has been used to demonstrate prognostic significance of CTC numbers in patients with metastatic breast, prostate, and colorectal cancers[2–7] (reviewed by Krebs et al.[8]) and subsequently has been approved by the US Food and Drug Administration as a prognostic biomarker and as an aid to monitoring treatment response in these disease types. We recently reported that CellSearch detection of ≥5 CTCs (per 7.5 ml of blood) in patients with advanced non-small cell lung cancer (NSCLC) is a poor prognostic factor and that a change in CTC number after a single cycle of standard-of-care chemotherapy predicts survival outcome.[9] However, two-thirds of patients with stage IV NSCLC had no detectable CTCs, and CTCs were detectable in less than 5% stage III patients using this technology platform.

Detection of CTCs using CellSearch is dependent on tumor cell expression of Epithelial Cell Adhesion Molecule

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(EpCam), an epithelial cell marker. However, the paradigm of epithelial to mesenchymal transition (EMT) as a predominant mechanism for tumor cell invasion and metastasis raises the possibility that not all cells in the circulation will express epithelial markers.[10–14] Thus, we hypothesized that the low prevalence of CTCs detected with the CellSearch technology in patients with advanced NSCLC, although strongly prognostic, may be due to the loss of EpCam expression. Therefore, we explored an antigen/EpCAM-independent approach for the ability to identify CTCs and for the potential to perform molecular characterization.

ISET(RareCell Diagnostics, Paris, France) is a filter-based, size exclusion technology capable of isolating CTCs independently of their expression of any particular marker. Using this technique, CTCs have been observed previously in patients with hepatocellular carcinoma, breast carcinoma, melanoma, and more recently in patients with early-stage, resectable NSCLC.[15–19] In a previous pilot study using ISET, we noted that CTCs were detectable in patients with advanced-stage NSCLC, with heterogeneity in both epithelial (E-cadherin and cytokeratin [CK]) and mesenchymal (N-cadherin and vimentin) marker expression.[20] In addition, circulating tumor microemboli (CTM; defined as clusters of cells with three or more nuclei) were identified, whereas CTM were not detected in our previous evaluation of tumor cells in 101 patients with NSCLC using CellSearch.[9]

Our goal was to compare the CellSearch (epithelial dependent) and ISET (antigen/EpCAM independent) platforms directly for the ability to isolate CTCs in a cohort of NSCLC patients. Secondary objectives were to explore the prevalence of CTM in NSCLC patients and to examine molecular characteristics of cells isolated by filtration, specifically in relation to their epithelial marker expression and proliferative status.

PATIENTS AND METHODS

Patient Eligibility and Clinical Characteristics

This was a prospective study conducted at the Christie Hospital, Manchester, United Kingdom. Patients with chemonäive, histologically proven NSCLC were eligible. Inclusion criteria included radiologically confirmed stage IIIA or IIIB/IV disease; World Health Organization performance status 0–2; age ≥18 years; and standard-of-care chemotherapy was to be administered. Patients with a history of prior malignancy, within the previous 5 years, were excluded. All patients provided written informed consent according to ethics board approved study protocols. Data were collected for age, ethnicity, histological subtype, stage of disease, smoking status, sites of metastases, and treatment received.

Blood Sampling

Peripheral blood (a maximum of 20 ml) samples were collected up to 1 hour before patients commencing their first cycle of chemotherapy. Blood (10 ml) was collected in a CellSave preservative tube (Veridex LLC, NJ), stored at room temperature, and processed according to standard Veridex protocols within 96 hours of collection. A further 8 to 10 ml was collected in an ethylenediaminetetraacetic acid tube (Becton Dickinson, NJ), stored at 4°C, and processed by ISET within 4 hours of collection. Samples were processed in accordance with the UK Clinical Trials regulations for compliance to Good Clinical Practice for laboratories.[21]

CTC Detection by CellSearch

Samples were analyzed as previously described.[22,23] A CTC was defined according to the criteria of round to oval morphology, cell size more than 4 μm, 4′,6-diamidino-2-phenylindole (DAPI) positive nucleus, CK positive staining, and absence of CD45 expression. CTC number is reported per 7.5 ml of blood. The sensitivity, accuracy, linearity, and reproducibility of the CellSearch system have been previously described.[22,24] Samples were considered positive for CTCs if ≥2 per 7.5 ml of blood were detected as one CTC has previously been reported as a normal finding.[22]

CTC Detection by ISET

Blood samples were divided into 1 ml aliquots and diluted 1:10 with red cell lysis buffer, containing 0.8% formaldehyde (RareCell Diagnostics), as per manufacturer's instructions. Each aliquot was placed into an individual well of a 10-well ISET filter module (consisting of a polycarbonate track-etched-type membrane punctured by 8-μm cylindrical pores, supported beneath a 10-well plastic reservoir).[15–18] Filtration was achieved by attaching the module to the ISET instrument and by applying regulated, gentle suction. Unfiltered cells were deposited on a 0.6-cm diameter, circular "spot" on the membrane beneath each well. After filtration, each spot was washed once with phosphate-buffered saline and then the membrane disassembled from the module and allowed to air dry at room temperature. Filters were subsequently stored at −20°C. The sensitivity of the ISET system has been previously reported as 1 cell per ml,[15,17,18] and similar results were found in our study (see Supplemental Table 1, http://links.lww.com/JTO/A198). Previous studies have reported that CTCs are not detectable in blood samples from healthy donors using the ISET technique.[18,19]

Identification of CTCs Isolated on ISET Filters

Tumor cells were identified according to absence of the leukocyte common antigen, CD45, by immunohistochemistry (IHC) and by their large, hyperchromatic, irregular-shaped nuclei. Individual spots (each representing 1 ml of filtered blood) were excised from the filter and placed in pH 6 antigen retrieval buffer (S1699, Dako) in a 99°C waterbath for 40 minutes. Spots were washed in tris-buffered saline, placed in 0.2% triton for 10 minutes to permeabilize cell membranes, and exposed for 30 minutes to 3% hydrogen peroxide–methanol solution to block endogenous peroxidases. Spots were subsequently washed in water and incubated overnight at 4°C with monoclonal mouse antihuman CD45 primary antibody (clone T29/33, Dako) diluted 1:30 in antibody diluent (S0809, Dako). CD45 staining was achieved with standard Envision Kits and the Liquid DAB+ Substrate Chromagen System according to manufacturer's instructions (K5007, Dako) and counterstained with Gill's hematoxylin. Spots were mounted on glass slides and scanned at 400× magnification using an Olympus BX52 microscope linked

