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ERCC1 expression in circulating tumor cells (CTCs) using a novel detection platform correlates with progression-free survival (PFS) in patients with metastatic non-small-cell lung cancer (NSCLC) receiving platinum chemotherapy

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ABSTRACT

Purpose: To utilize a novel circulating tumor cell (CTC) technology to quantify ERCC1 expression on CTCs and determine whether ERCC1 expression levels predict efficacy of platinum-based chemotherapy in patients with metastatic non-small-cell lung cancer (NSCLC).
Experimental design: ERCC1 expression was measured in 17 metastatic NSCLC patients who received platinum-based therapy and had ≥2 intact CTCs with acceptable ERCC1 expression assay results. ERCC1 levels were determined from average expression on individual CTCs in each sample. Progression-free survival (PFS) was calculated from the date of therapy initiation.
Results: PFS decreased with increasing ERCC1 expression ($p < 0.04$, *F*-test, linear regression). Lack of ERCC1 expression was associated with longer PFS (266 days versus 172 days, log-rank, $p < 0.02$) in a Kaplan–Meier analysis using ERCC expression level of 1 as a cutoff (range 0–30). The difference in survival was statistically significant with a hazard ratio of 4.20 (95% CI 1.25–14.1, $p < 0.02$, log-rank). PFS was also observed to decrease with increased cytokeratin (CK) expression ($p < 0.01$ long-rank (Cox regression) and *F*-test (linear regression)). The hazard ratio is 4.38 (95% CI 1.76–10.9) for each log-change in CK value until progression was noted on imaging.
Conclusion: Low expression of ERCC1 on CTCs correlates with PFS in patients with metastatic NSCLC receiving platinum-based therapy.

1. Introduction

Non-small cell lung cancer (NSCLC) represents over 80% of all cases of lung cancer and is the leading cause of cancer-related death in the US [1], with median survival in metastatic disease of less than 1 year. To improve the effectiveness of available treatments and survival for these patients, there are ongoing attempts to "personalize" treatment, by selecting therapy based upon an individual patient's tumor molecular profile. Current challenges to personalizing treatment include a lack of adequate biomarkers and difficulty in obtaining tumor samples of sufficient size and quantity for molecular analysis in non-surgical patients. The increasing use of fine needle and bronchoscopic biopsy results in small specimens such that secondary analysis is not possible in many lung cancer patients [2].

Circulating tumor cells (CTCs) are present in the peripheral blood of patients with metastatic cancer and have been evaluated both quantitatively and qualitatively in various tumor types. The number of CTCs is an independent prognostic factor and can be used to monitor treatment efficacy [3–6]. Success with locating CTCs in patients with NSCLC has been limited, with fewer than 20% of patients with metastatic lung cancer having a positive test using current methods of CTC detection [7]. Most methods of CTC detection rely upon an enrichment step utilizing epithelial cell adhesion molecule (EpCAM), which is expressed at lower levels in CTCs compared to normal tissues, leading to decreased sensitivity [8,9]. EpCAM expression is also highly variable between tumor types [10,11]. Furthermore, the CTC images produced by these

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methods often lack the fidelity needed for quantitative evaluation of biomarker expression levels.

Available technologies for CTC detection have enabled profiling of CTCs for biomarkers such as human epidermal growth factor receptor-2 (HER2) for the purpose of guiding therapy in breast cancer patients. Notably, discordance has been reported to be substantial, with up to 50% discordance in early and late HER2-negative patients [12–14]. These results pose the possibility that biomarkers on CTCs provide a more accurate representation of the present state of disease.

The excision repair cross-complementation group 1 (ERCC1) enzyme removes cisplatin-induced DNA adducts and is a potential biomarker for predicting response to platinum therapy. In lung cancer patients with stage IIIB/IV disease, low levels of ERCC1 expression correlate with platinum sensitivity and prolonged overall survival (OS) [15–17]. A randomized phase III trial customizing chemotherapy in patients with metastatic NSCLC based upon pretreatment ERCC1 mRNA levels showed that patients in the genotypic arm who had low ERCC1 expression and were assigned to receive cisplatin/docetaxel had an improved ORR (50.7%) compared to those patients in the control arm (39.3%) who received cisplatin/docetaxel [18]. Another phase III trial in patients with advanced NSCLC found that ERCC1 values were significantly and inversely correlated with disease response [19]. Low expression of ERCC1 expression in tumor tissue also correlates with a progression-free survival (PFS) benefit in patients with stages I–III NSCLC receiving adjuvant chemotherapy with a platinum doublet [20]. Whether ERCC1 expression in NSCLC CTCs correlates with clinical outcome is unknown.

We previously reported a novel platform of CTC detection with a high-speed scanning instrument utilizing fiberoptic array scanning technology that enables accurate location of individual CTCs on a large substrate without the need for sample enrichment [21]. The scanner locates cells with cytokeratin (CK) expression, which is also used to identify them. In contrast, various enrichment approaches involve the attachment of magnetic particles to EpCAM expression on the cell surface for separation of CTCs from the sample using magnetic fields [3,22] or alternatively the capture of CTCs on the surface of microfluidic devices by EpCAM receptors [23,25]. These enrichment approaches can miss CTCs that have low levels of EpCAM expression [10,11]. The location accuracy of our platform enables high quality imaging with automated microscopy for quantification of protein biomarkers, such as ERCC1 [26]. Here we present findings of ERCC1 expression on CTCs using this detection method.

2. Methods

2.1. Inclusion criteria

Patients with NSCLC from Stanford and Billings who fit eligibility criteria were enrolled into the study between January 2009 and June 2010. The patients provided written, voluntary, informed consent to IRB-approved protocols. Patients enrolled at Stanford provided one sample at diagnosis before starting first-line platinum-containing therapy or at the time of progression. Patients enrolled at Billings provided samples at the time of diagnosis and at 3-month intervals thereafter, for a period of 1 year. The cohort includes only the earliest sample from each patient that satisfies the inclusion criteria. PFS is measured from start of platinum-based therapy to tumor progression, as determined by the date of imaging study performed when the radiologist report noted progression.

2.2. Sample preparation

Ten milliliter samples of blood were drawn, shipped and plated on large glass substrates as described elsewhere [24]. Labeling for CTC identification with CK, DAPI and CD45 has been previously described [24]. The mouse monoclonal anti-human IgG2b ERCC1 (sc-17809, Santa Cruz Biotech) was included in the cocktail of other primary antibodies. The secondary antibody for ERCC1 was goat anti-mouse IgG2b (A-21242, Invitrogen) tagged with Alexa 647. Staining quality was evaluated by measuring stain localization and contrast, and by assessing the difference between the staining level of moderate expressing control cells and the staining level of leukocytes, the negative control.

2.3. CTC imaging and identification

A cytometer using fiber array scanning technology scanned the glass slide containing the processed sample at 25M cells/min, and emission from CK labeling was collected by an array of optical fibers forming a wide collection aperture and detected by a filtered photomultiplier [21]. The instrument located potential CTCs to within 40 μm, allowing subsequent high resolution imaging with an automated digital microscope (ADM). Autofluorescence was monitored in a separate detector and was used to eliminate nearly all non-labeled objects. The morphology of the cells was well preserved during preparation.

Cytokeratins were the primary positive markers for identification of CTCs. While different types of epithelial cells express different CK subtypes, CK is universally expressed in epithelial cells, and a mixture of CK isoforms ensures broad coverage [27]. A negative control (CD45) was used to ensure against nonspecific binding and native fluorescence from granulocytes. To be evaluated for ERCC1, targets were required to possess an intact nucleus and CK staining patterns with no apoptotic features and also to be CD45 negative.

2.4. ERCC1 scoring

The localization of ERCC1 expression in the nucleus and the ratio of expression level in control cells to negative controls were used to monitor labeling quality. Only samples with ERCC1 signal localized to the nucleus and a signal ratio of 2:1 between control cells and negative controls were included. CTCs that exhibited blebbing, an indication of apoptosis, were not included in the analysis. ERCC1 expression is reported as average signal intensity over the nuclear region. Note that CK signal in the nuclear region was typically much lower than in the cytoplasm, so the ERCC1 expression is not due to bleed-through from the CK expression.

Biomarker signal quantification was performed using internally developed software. Digital analysis and visual evaluation was used for scoring. Analogous with tissue analysis, the scoring methodology combined expression level with the percentage of biomarker-positive cells [20,28–30]. Each cell in the sample was scored as either 0, 1, 2 or 3, depending on whether its ERCC1 expression was equal to or lower than negative controls (leukocytes, score = 0), between the negative and positive controls (score = 1), similar to the positive controls (score = 2), or higher than the positive controls (score = 3). The sample score was calculated by taking the average CTC score and multiplying by ten. Averaging accounts for the percentage of cells expressing the biomarker. A patient score one or above was considered “high” in our analysis.

A-549 cells were used as a positive control for ERCC1 [31,32]. A protocol for CTC identification and biomarker analysis was established and refined through independent assessments and consensus between two personnel (RHB and JL) with inputs from two pathologists.