#7352

Characterization of ALK Fusions in Circulating Tumor Cells (CTCs) of NSCLC

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Background

As many as 40% of non-small cell lung cancer (NSCLC) adenocarcinoma patients have insufficient tumor sample or have high co-morbidities preventing access to tissue biopsies for FISH or IHC to identify an ALK fusion and subsequent eligibility for Crizotinib therapy. This unmet medical need could be mitigated by the development of a fluid biopsy that can characterize patient blood for the presence of ALK-driven NSCLC. We developed a gene/protein assay for the ALK fusion and molecularly characterized CTCs and CTC subpopulations in newly diagnosed NSCLC patients. In addition to "traditional" CTCs with epithelial morphology, CTC subpopulations that include apoptotic, small, and cytokeratin (CK)-negative cells have been identified as CTCs in mCRPC (ASCO GU 2014) via assessment of PTEN/ERG alterations. The Epic platform enables the detection of NSCLC CTCs with unique morphology and epithelial plasticity. We set out to measure the frequency of CTCs and identify CTC subpopulations (traditional, small or CK-) in NSCLC, and to further develop a gene/protein assay to assess ALK status of NSCLC patients with confirmed ALK+ or ALK- lung biopsies by FISH analysis.

Methods

Ten newly diagnosed NSCLC patients were recruited prior to therapy, and blood specimens were collected and shipped to Epic Sciences. 2/10 had known ALK rearrangement through FISH analysis performed on tissue biopsies. All nucleated cells were plated onto glass slides and subjected to immunofluorescence (IF) staining and CTC identification by fluorescent scanners and algorithmic analysis. CTCs, defined as traditional (CK+, CD45- with intact DAPI nuclei and are morphologically distinct), apoptotic (CK+, CD45-, non-intact nuclei) and CK- (CK-, CD45-, intact and distinct nuclei) were identified. Samples were characterized with ALK IF to assess protein expression. Patients with known ALK rearrangements in tissue also had their CTCs assessed by Epic's ALK FISH assay.

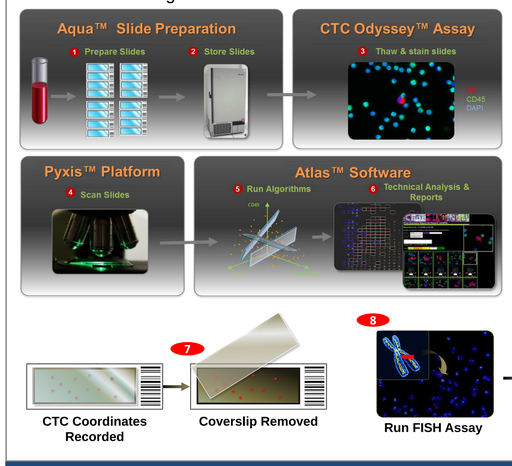

Figure 1: Schematic of Epic's CTC collection and detection process: 1) Nucleated cells from blood sample are placed onto slides; **2)** Slides stored in -80C biorepository; **3)** Slides stained with CK, CD45, DAPI and ALK; **4)** Slides scanned; **5)** Multi-parametric digital pathology algorithms run; **6)** Software and human reader confirmation of CTCs & quantitation of biomarker expression; **7)** For FISH, coordinates are recorded and coverslip removed; **8)** FISH assay is run; **9)** Regional WBCs are scored to assess normal; **10)** CTCs relocated and scored.

ALK Protein Assay Development

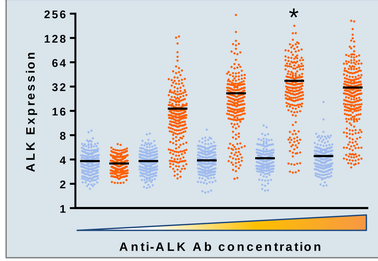

Figure 2: Antibody titration curve of a rabbit monoclonal anti-ALK antibody. Increasing concentrations of an anti-ALK rabbit monoclonal antibody were applied to either H2228 (ALK fusion, orange) or A549 (intact ALK, blue) cells to generate a titration curve. The scatter plot indicates relative ALK protein expression quantified on each cell detected on the Epic platform (black bars denote mean expression). At the optimal antibody concentration (*), the average ALK signal measured in H2228 cells is 10-fold higher than that in A549 cells.

NSCLC CTC Subpopulations Detected on Epic Platform

Representative images of CTCs with various biomarker profiles detected in NSCLC patient blood.

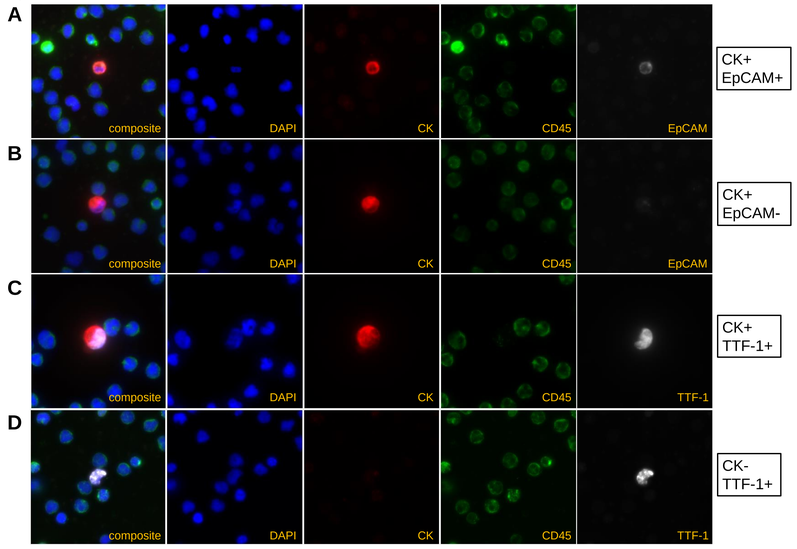

Figure 3: CTCs found in NSCLC patient blood on the Epic Platform have diverse biomarker expression profiles. NSCLC CTCs can be cytokeratin (CK)+/EpCAM+ **[A]** or CK+/EpCAM- **[B]**, demonstrating plasticity in epithelial marker expression. Furthermore, CTCs from patients with confirmed adenocarcinoma (ADC) can be CK+/TTF1+ **[C]**, CK+/TTF1- (not shown) and CK-/TTF1+ **[D]**, with the latter demonstrating a case wherein a CTC has potentially undergone EMT, losing epithelial morphology. The Epic Platform enables detection and biomarker quantitation independent of specific epithelial markers.

ALK Protein & Gene Assay

Assays for ALK protein were developed and specificity was confirmed utilizing H2228 (ALK+) and A549 (ALK-) cells spiked into healthy donor blood.

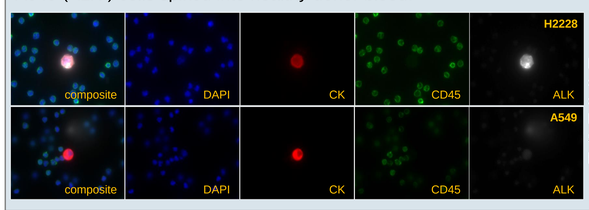

Figure 4: Representative images of H2228 (top) and A549 (bottom) cell line cells stained using the Epic protein assay demonstrate specificity and sensitivity for ALK protein expression.

With the Epic Platform, individual CTC protein expression can be quantified and immediately followed by genomic profiling utilizing FISH.

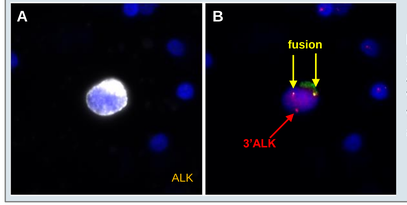

Figure 5: ALK protein expression corresponds with ALK gene rearrangement in H2228 cells. Following staining for ALK protein **[A]**, individual cells were relocated and assayed via FISH **[B]**. ALK rearrangement was determined by visualizing deletion of 5'ALK (**red** arrow). Intact ALK is seen as an adjacent red/green or yellow signal (**yellow** arrows).

Clinical Feasibility

ALK+ CTCs were found in blood samples from 1 of 2 patients with confirmed ALK rearrangement (assessed via biopsy), which contained both CK+/ALK+ and CK-/ALK+ cells.

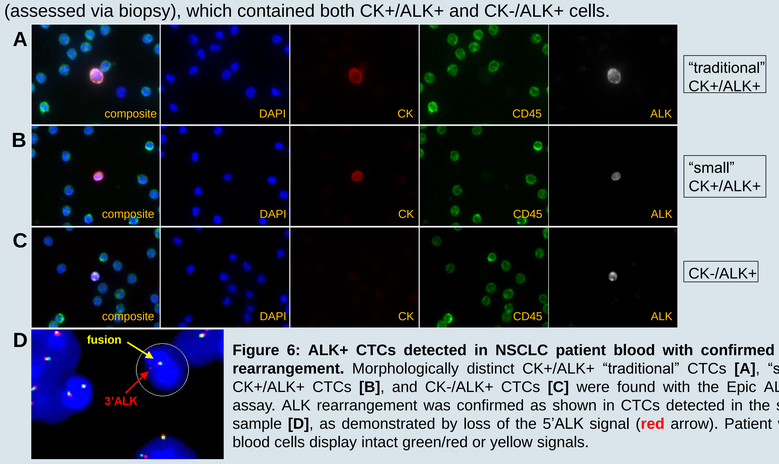

Figure 6: ALK+ CTCs detected in NSCLC patient blood with confirmed ALK rearrangement. Morphologically distinct CK+/ALK+ "traditional" CTCs **[A]**, "small" CK+/ALK+ CTCs **[B]**, and CK-/ALK+ CTCs **[C]** were found with the Epic ALK IF assay. ALK rearrangement was confirmed as shown in CTCs detected in the same sample **[D]**, as demonstrated by loss of the 5'ALK signal (**red** arrow). Patient white blood cells display intact green/red or yellow signals.

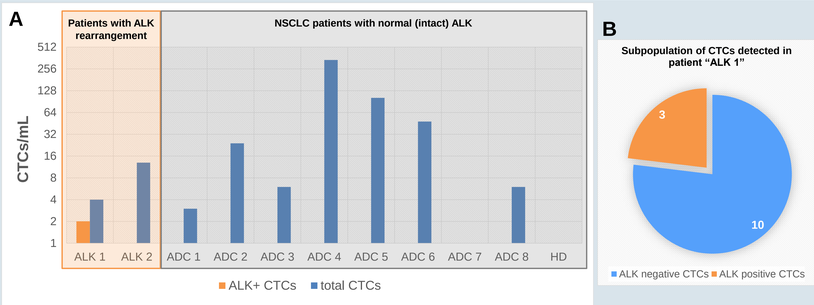

Figure 7: Enumeration of total and ALK+ CTCs in patient samples. [A] Samples from ten confirmed NSCLC patients and one healthy donor were assessed with the Epic ALK assay. CTCs expressing ALK protein were found in 1 of 2 ALK rearranged patient samples (ALK 1, orange bar). No ALK+ CTCs were detected in 8/8 adenocarcinoma patients with normal ALK status (ADC 1-8). The healthy donor sample contained no detectable CTCs (HD). **[B]** In patient sample ALK 1, 3 ALK+ and 10 ALK- CTCs were found in approximately 3 mL of patient blood. Any CTC with ALK protein expression >12-fold higher than background was determined to be ALK+. The ALK 1 patient sample was found to contain ALK rearranged CTCs as assessed by FISH (Fig. 6D).

Conclusions

Epic's ALK fusion protein assay and ALK FISH analysis demonstrate both sensitivity and specificity, potentially enabling the identification of patients eligible for Crizotinib. The Epic Platform enables the detection of traditional, small and EMT (CK- and/or EpCAM-) CTCs in NSCLC, allowing for the detection of ALK in CTC subpopulations with unique cell morphology and epithelial plasticity (i.e., lacking specific epithelial markers). Further clinical studies are ensuing to determine the clinical value of Epic's ALK assay and the relevance of these CTC subpopulations to determining Crizotinib therapy and NSCLC progression. We are also establishing methods for detailed genomic analysis of ALK+ CTCs with various morphologies inclusive of both FISH and single-cell genomics.

These studies will further validate the clinical utility of a specific ALK protein assay using the Epic CTC platform to identify patients eligible for Crizitonib, especially in those that may be ineligible for biopsy due to co-morbidity or tumor inaccessibility.