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Expanded CTCs from a Patient with *ALK* Positive Lung Cancer Present *EML4-ALK* Rearrangement along with Resistance Mutation and Enable Drug Sensitivity Testing: A Case Study

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Abstract

The emergence of liquid biopsy using circulating tumor cells (CTCs) as a resource to identify genomic alterations in cancer presents new opportunities for diagnosis, therapy and surveillance. The presented study identified *EML4-ALK* gene rearrangement in expanded CTCs from one *ALK* positive lung adenocarcinoma patient. At the time of radiographic progression, CTCs obtained from the patient revealed a drug resistance mutation, L1196M on the *ALK* gene. CTCs were expanded *ex-vivo* and drug sensitivity testing was performed using 2 ALK inhibitors, crizotinib and ceritinib. The half maximal inhibitory concentration (IC50) of ceritinib was 1664 nM compared with crizotinib, 2268 nM showing that ceritinib was a more potent ALK inhibitor. We demonstrate that it is feasible to detect serial genetic alterations in expanded CTCs and perform *in vitro* drug screening. These findings support the clinical utility of CTCs not only for diagnosis, but also a potential tool for drug sensitivity testing in distinct subsets of lung cancer and for personalized precision medicine.

Introduction

Advances in sequencing have shifted the landscape of non-small cell lung cancer (NSCLC) therapy to a personalized approach that is driven by molecular alterations in each patient's tumor, leading to improved survival for a small minority of patients. 3–7% of NSCLC

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patients have tumors that contain an inversion in chromosome 2 that juxtaposes the 5′ end of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene with the 3′ end of the anaplastic lymphoma kinase (*ALK*) gene, resulting in the novel fusion oncogene *EML4-ALK*[1]. Therapy in this group of patients with a specific tyrosine kinase inhibitor, crizotinib, resulted in rapid (within 6 weeks), greater response rates (65%) and longer progression free survival (PFS 7.7 months), compared with chemotherapy[2]. However, most patients with *ALK*-positive lung cancer who respond to crizotinib undergo a relapse within months (3.8–21 months) due to emergence of resistance mechanisms[3]. Among the various resistance mechanisms, gatekeeper mutation in the *ALK* gene (L1196M) is identified in 9% of patients[4]. The second-line inhibitor, ceritinib, overcomes this crizotinib-resistant mutation. More recently, third generation ALK inhibitors with improved IC50, targeting wild-type *EML4-ALK* as well as resistance mutations are in clinical trials, including PF-06463922[5].

To enhance treatment response, it is imperative to identify the most effective tyrosine kinase inhibitor at the outset as well as identify, by longitudinal testing the emergence of resistance which typifies the molecular evolution of these driver oncogene mediated cancers. At the current time, the practice is to biopsy and re-biopsy at the time of clinical/radiographic progression, which is invasive and not without risk. *ALK* rearrangement is identified through fluorescence *in situ* hybridization (FISH) using a break-apart probe[6]. A separation of a red and a green signal indicates gene rearrangement. In recent years, *EML4-ALK* rearrangement has been detected in circulating tumor cells (CTCs) from peripheral blood of NSCLC cancer patients[7, 8]. CTCs can be expanded *ex vivo* for genotyping and drug testing, as demonstrated in breast and colon cancer[9, 10].

Here we present a case study on one advanced stage NSCLC cancer patient harboring *EML4-ALK* rearrangement in the tumor. CTCs were isolated from the peripheral blood of this patient during three clinical visits. The cells were expanded through a microfluidic co-culture model reported in our previous study[11]. We found concordant *ALK* rearrangement and secondary resistance mutation L1196M in the CTCs similar to that in the tumor. Drug testing with crizotinib and ceritinib was also performed on the expanded CTCs.

Results

Primary tumor mutation status

A 42-year-old man with a history of Stage IV Hodgkin's lymphoma was found to have metastatic lung adenocarcinoma on re-staging scans (Figure 1A). Biopsy of the lung mass and FISH revealed *ALK* gene rearrangement (Figure 1B). The patient was initially placed on crizotinib. A computerized tomography (CT) scan 4 months after crizotinib revealed near complete resolution of disease. However, a scan at 7 months showed progression in the right hilum (Figure 1A). Patient underwent a bronchoscopy and re-biopsy of the hilar mass. Whole-exome sequencing (70–100× coverage) of the biopsy revealed a secondary mutation L1196M and resultant resistance to crizotinib. He was then treated with ceritinib and his repeat scans revealed a marked decrease in the hilar mass. The patient had serial imaging of the brain with magnetic resonance imaging (MRI). At the time of first disease progression, that was 2 months prior to start of ceritinib treatment, the brain MRI was negative. The next brain imaging occurred when he had neurological symptoms. The patient then had regular

brain surveillance with MRI. After 11 months, the patient had a progression of disease with brain metastasis in addition to progression in right lower lobe and right hilum. Whole-exome sequencing of the biopsy of brain metastasis revealed wild-type *EML4-ALK* and an absence of L1196M mutation. ALK amplification and alternative oncogenic drivers were not detected in the brain biopsy. Table 1 summarizes the mutation status of tumor biopsy at three clinical visits.

Detecting ALK rearrangement in expanded CTCs

In parallel, we isolated and expanded CTCs from his blood at the time of initial diagnosis and at the time he developed resistance to crizotinib and then brain metastasis. FISH analysis identified *ALK* rearrangement in expanded CTCs (Figure 2). At initial diagnosis, 83 cells were counted, 15 cells had 1 copy, 6 cells had 2 copies and 4 cells had 3 or more copies of *ALK* (30% cells positive). At the time when patient developed resistance to crizotinib, 54 cells were counted, 5 cells had 1 copy, 3 cells had 2 copies of *ALK* (15% cells positive). When patient developed brain metastasis, 86 cells were counted, 10 cells had 1 copy, 2 cells had 2 copies and 2 cells had 3 or more copies of *ALK* (16% cells positive). The criteria for ALK positivity is at least 15% cells containing separated signals with at least 50 cells counted[12]. Accordingly, CTCs were positive for *ALK* rearrangement at all 3 time points, concordant with findings on tumor biopsies (Table 2).

Identifying L1196M mutation in expanded CTCs

After examining *ALK* positivity, we performed Sanger sequencing to detect L1196M mutation on the *ALK* gene. It was found that the gatekeeper mutation emerged in the expanded CTC when the patient developed resistance to crizotinib and had radiographic progression. The L1196M mutation disappeared from expanded CTCs collected at the time of brain metastasis, which again mirrored the findings from the brain biopsy (Figure 2, Table 2).

Drug testing on expanded CTCs

After verifying mutational status of expanded CTCs, we performed *in vitro* experiments to test CTCs collected and cultured at initial visit against crizotinib and ceritinib. As the CTCs were admixed with cancer-associated fibroblasts (CAFs), we also conducted control experiments on pure CAFs, A549 (resistant negative control) and H3122 (*ALK* rearranged, sensitive positive control for crizotinib and ceritinib) cell lines. The IC50s to the 2 compounds, using the expanded *ALK* rearranged CTCs are shown in Figure 3. The IC50 for crizotinib and ceritinib was 2268 nM and 1664 nM respectively.

Discussion

We previously reported a 3D microfluidic co-culture model for expanding small number of CTCs in lung cancer patients[11]. The present study demonstrates that expanded CTCs carry *ALK* rearrangements and dynamic detection of drug-resistant mutations by analyzing CTCs longitudinally is possible. The emergence and disappearance of L1196M mutation likely reflects the clonal evolution of tumor cells toward drug resistance against crizotinib and

ceritinib. Through expansion of CTCs, drug-susceptibility testing was enabled, offering unique advantages over serum markers and circulating cell-free nucleic acids.

Uniquely, we demonstrate the feasibility of performing drug testing on expanded lung CTCs, which is a challenge due to small numbers of the cells. Performing drug screening by isolating CTCs from peripheral blood is meaningful because it is less invasive than solid tumor biopsy and more accessible. In this study, the IC50 for crizotinib was higher than ceritinib (2268 nM v 1664 nM), as reported by others[4]. These values were higher than reported IC50s against the H3122 cell line, likely due to the presence of CAFs. However, the ratio of IC50 favored ceritinib as the more potent ALK inhibitor against *ALK*-rearranged CTCs.

Personalized drug testing predicts therapeutic responses especially in lung cancer. It was reported that 64% of driver mutations were detected in patients with lung adenocarcinoma, of which nearly 30% were selected for targeted therapies[13]. It is imperative to monitor acquired mutations rendering targeted therapies ineffective early in disease progression. CTCs as “liquid biopsy” can potentially serve as drug testing platforms to guide therapeutic selection.

Materials and Methods

CTC isolation and expansion

Five mL of peripheral blood was drawn from the lung cancer patient into purple top EDTA tubes during each visit. Blood was drawn at University of Michigan Hospital under an IRB-approved protocol. CTCs were captured with a cocktail of antibodies against EpCAM and CD44 in three CTC-capture devices and cultured with CAFs in the devices for two weeks and then outside devices for one month. The detailed procedure can be found in our previous study[11].

FISH analysis on CTCs

CTCs were spun onto polylysine-coated slides, and fixed with 1:3 acetic acid and methanol followed by air-drying. FISH test with two-color and break-apart probes were performed on the slides. The probes used were green 5′ ALK probe (RP11-993C21) and red 3′ ALK probe (RP11-984I21). Co-localizing red and green signal indicated normal chromosome and separate red and green signal indicated *ALK* rearrangement. All images were captured under 100× oil immersion objective using Zeiss Axioplan microscope equipped with CCD camera. Captured images were processed using In situ Imaging System (ISIS) software (Metasystems, Germany).

Drug testing

1000 CTCs, CAFs, A549, or H3122 were plated in each well on a 96-well plate. Each drug concentration had wells in triplicate. Six different concentrations were tested for each drug. Cells were incubated with drugs for 72hrs. After treatment, each well was incubated with Cell Proliferation Reagent WST-1 assay (Roche). Absorbance was measured with Biotek-

Synergy Neo-plate Reader. IC50s were determined by nonlinear regression model by Prism Graphpad.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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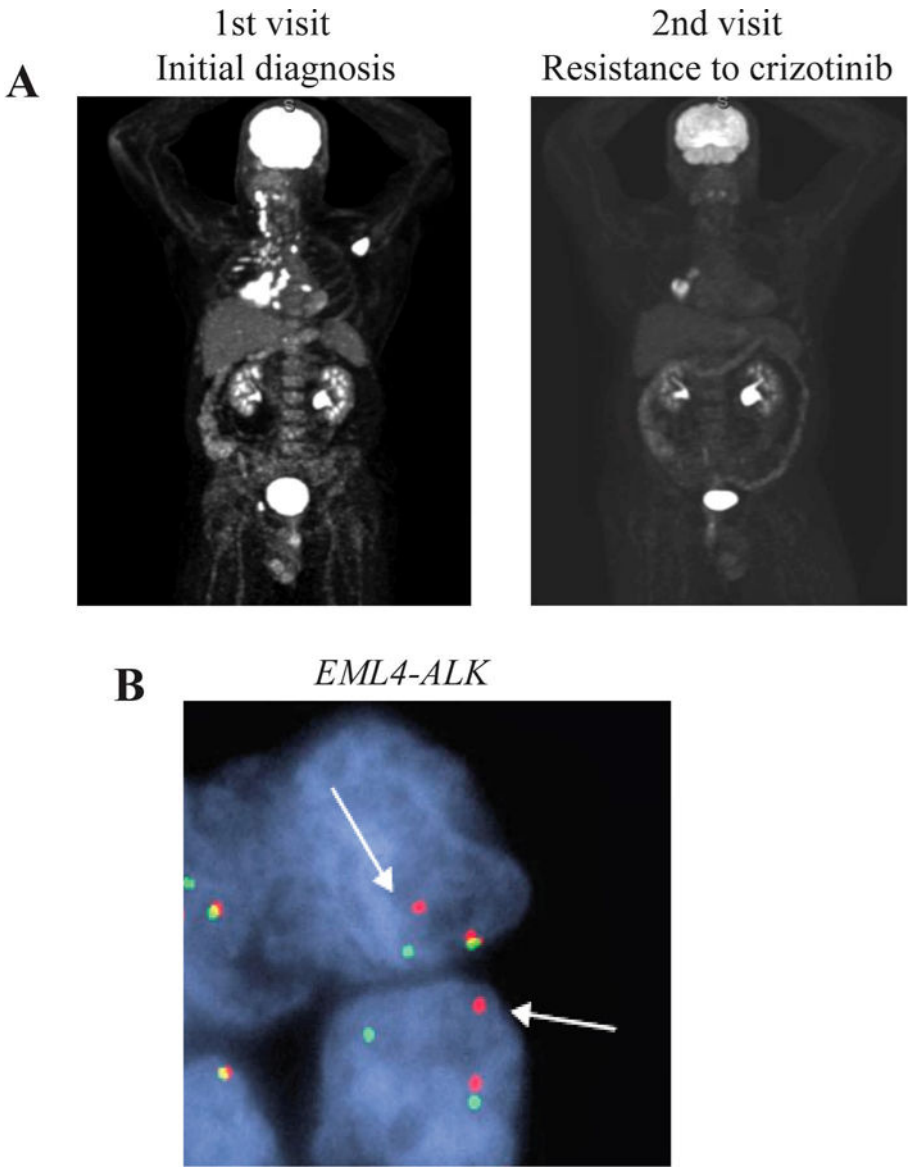


Figure 1. Tumor status of the patient

(A) Computed tomography scan at initial diagnosis and when the patient was resistant to crizotinib. (B) *EML4-ALK* rearrangement is detected in the tumor biopsy specimen at initial visit by FISH (1000× magnification).

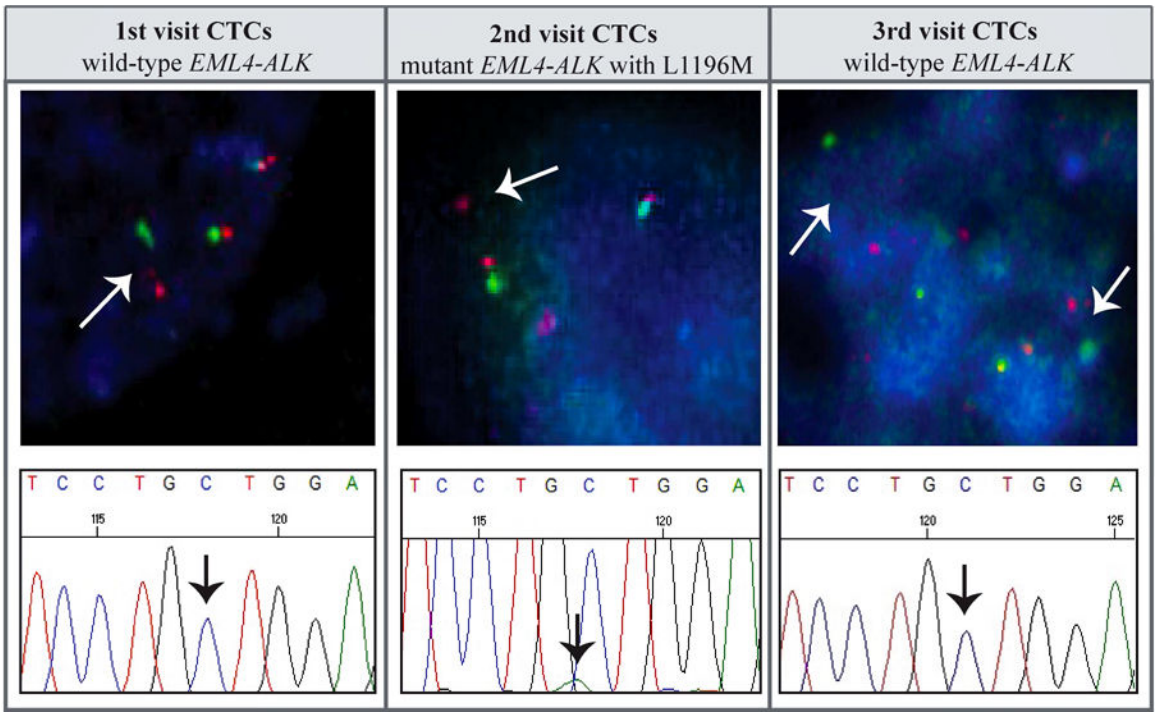
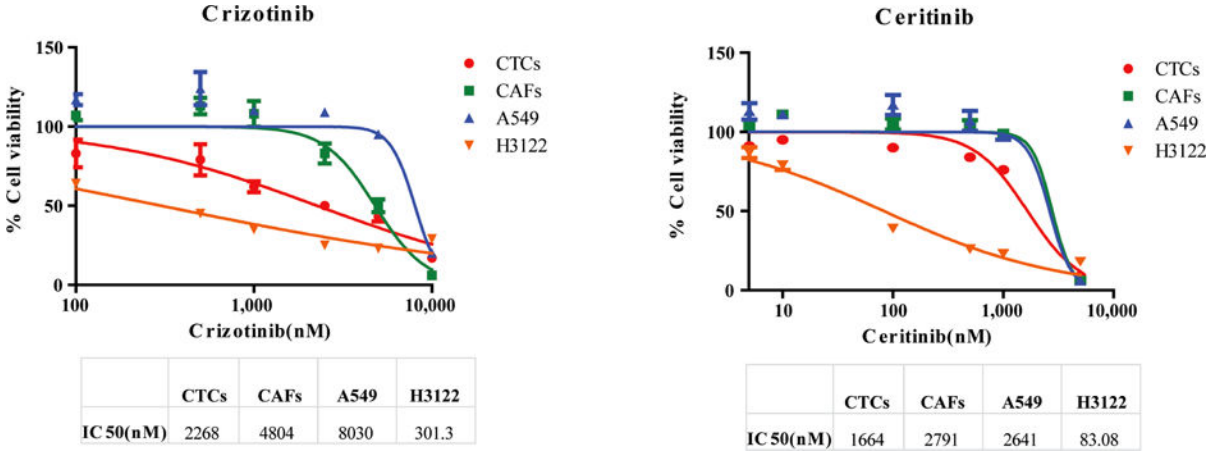


Figure 2. Mutation analysis of expanded CTCs

Expanded CTCs, during the three clinical visits, are positive for *EML4-ALK* rearrangement by FISH (1000× magnification). DNA sequencing reveals L1196M mutation in expanded CTCs when the patient developed resistance to crizotinib (2nd visit) and an absence of the mutation when brain metastasis occurred (3rd visit).



	CTCs	CAFs	A549	H3122
IC 50(nM)	2268	4804	8030	301.3

	CTCs	CAFs	A549	H3122
IC 50(nM)	1664	2791	2641	83.08

Figure 3. IC50s of cells treated with crizotinib and ceritinib

Expanded CTCs are compared with pure CAFs, a resistant cell line A549 and a sensitive cell line H3122.

Table 1

Mutation status of primary tumor at 3 time points

PRIMARY TUMOR	1st visit	2nd visit	3rd visit
Clinical time point	Initial diagnosis	Resistance to crizotinib	Brain metastasis
Tumor biopsy site	Lymph node	Right hilum	Brain
Gene fusions	*EML4-ALK*	*EML4-ALK*	*EML4-ALK*
Somatic point mutations	N/A	ALK (L1196M) gatekeeper mutation- confers resistance to crizotinib	No ALK (p.L1196M) gatekeeper mutation detected

Table 2

Mutation status of expanded CTCs at 3 time points

CTCs	1st visit	2nd visit	3rd visit
Clinical time point	Initial diagnosis	Resistance to crizotinib	Brain metastasis
Gene fusions	*EML4-ALK*	*EML4-ALK*	*EML4-ALK*
L1196M	No	Yes	No