A Prospective Evaluation of Circulating Tumor Cells and Cell-Free DNA in EGFR-Mutant Non-Small Cell Lung Cancer Patients Treated with Erlotinib on a Phase II Trial

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Abstract

Purpose: Genotype-directed therapy is the standard of care for advanced non–small cell lung cancer (NSCLC), but obtaining tumor tissue for genotyping remains a challenge. Circulating tumor cell (CTC) or cell-free DNA (cfDNA) analysis may allow for noninvasive evaluation. This prospective trial evaluated CTCs and cfDNA in EGFR-mutant NSCLC patients treated with erlotinib until progression.

Experimental Design: EGFR-mutant NSCLC patients were enrolled in a phase II trial of erlotinib. Blood was collected at baseline, every 2 months on study, and at disease progression. Plasma genotyping was performed by droplet digital PCR for EGFR19del, L858R, and T790M. CTCs were isolated by CellSave, enumerated, and analyzed by immunofluorescence for CD45 and pan-cytokeratin and EGFR and MET FISH were also performed. Rebiopsy was performed at disease progression.

Results: Sixty patients were enrolled; 44 patients discontinued therapy for disease progression. Rebiopsy occurred in 35 of 44 patients (80%), with paired CTC/cfDNA analysis in 41 of 44 samples at baseline and 36 of 44 samples at progression. T790M was identified in 23 of 35 (66%) tissue biopsies and 9 of 39 (23%) cfDNA samples. CTC analysis at progression identified MET amplification in 3 samples in which tissue analysis could not be performed. cfDNA analysis identified T790M in 2 samples in which rebiopsy was not possible. At diagnosis, high levels of cfDNA but not high levels of CTCs correlated with progression-free survival.

Conclusions: CTCs and cfDNA are complementary, noninvasive assays for evaluation of acquired resistance to first-line EGFR TKIs and may expand the number of patients in whom actionable genetic information can be obtained at acquired resistance. Serial cfDNA monitoring may offer greater clinical utility than serial monitoring of CTCs.

Introduction

Tumor genotyping has emerged as the standard of care for patients with newly diagnosed non–small cell lung cancer (NSCLC) because of the efficacy of targeted therapies for patients whose tumors are ultimately found to have activating mutations in specific oncogenic drivers (1). However, despite the initial efficacy of tyrosine kinase inhibitors (TKI) targeting sensitizing mutations in EGFR (2–4) or rearrangements in ALK or ROS1 (5, 6), acquired resistance is inevitable (7, 8). The most common mechanism of acquired resistance to first-line EGFR TKIs is the EGFR T790M mutation (7, 8), and in November 2015, osimertinib became the first investigational third-generation EGFR TKI to receive FDA approval for patients with an acquired T790M mutation at the time of disease progression (9). Notably, the clinical use of these third-generation inhibitors requires repeat tumor biopsy at the time of disease progression to identify mechanisms of acquired resistance. Biopsy of an advanced solid tumor can be associated with increased patient morbidity; thus, the parallel development of advanced technologies for noninvasive liquid biopsies, analysis of circulating tumor cells (CTC; ref. 10) or cell-free plasma DNA (cfDNA; ref. 11), represents potential adjunctive or alternative method(s) for identifying targetable mechanisms of acquired resistance in patients with progression on first-line TKIs.

CTCs can be identified in blood samples from patients with many different types of cancer, including lung cancer (12). Ongoing research efforts focus not only on improving analyses of these circulating single cells but also on identifying prognostic and therapeutic applications within the treatment context of a specific cancer. In NSCLC, CTCs have been detected in the plasma at different time points throughout disease treatment and in patients with different stages of disease (13–15). However, the prognostic
Translational Relevance

Ongoing research efforts to elucidate the mechanism(s) of acquired resistance to targeted therapies in patients with non-small cell lung cancer (NSCLC) have led to emerging new strategies to target acquired resistance. However, some recently approved drugs, such as osimertinib for the acquired EGFR T790M resistance mutation, require repeat biopsy for genomic testing. Although analyzing tumor tissue remains the gold standard, noninvasive biopsy methods, including evaluation of circulating tumor cells (CTC) or cell-free DNA (cfDNA), represent a novel way of detecting specific oncogenic mutations or following genomic changes within a tumor over time without the need for a repeat tissue biopsy. This prospective trial evaluated CTCs and cfDNA at baseline, at progression, and throughout treatment in patients with EGFR-mutant NSCLC treated with erlotinib until disease progression and compared the outcomes with repeat tissue biopsy to more fully evaluate and compare the clinical utility of these noninvasive biopsy methods with tissue biopsy.

significance of isolating and quantifying CTCs and/or observing temporal changes from an individual patient remains incompletely understood (16, 17). Mutations or rearrangements in EGFR or ALK have been identified in circulating cells from some NSCLC patients (18–20), suggesting that evaluation of CTCs could represent a noninvasive way to follow tumor response and assess acquired genomic changes in advanced NSCLC over time during treatment with targeted therapies.

Similarly, sequencing of cfDNA has been used to identify activating mutations and acquired resistance mutations in EGFR (19, 21), monitor changes in cfDNA throughout treatment (22), and evaluate cfDNA as a prognostic or predictive biomarker in NSCLC patients (23, 24). cfDNA evaluation has also been used to identify a novel mechanism of acquired resistance to the third-generation EGFR T790M inhibitor osimertinib, specifically the emergence of the acquired EGFR C797S mutation (25). An ongoing clinical trial is now validating prospective cfDNA testing as a diagnostic tool based upon the identification of targetable genomic alterations (clinical trial registration ID, NCT02279004).

Both invasive and noninvasive biopsy methods offer advantages and disadvantages for evaluating and monitoring disease in NSCLC, but limited data are available to directly compare the utility of tissue biopsy, CTC analysis, and cfDNA analysis in the same patient cohort in a prospective setting. Accordingly, a phase II study of first-line erlotinib in patients with known EGFR-activating mutations was carried out in patients prospectively consented to repeat tumor biopsy at the time of disease progression along with baseline, serial, and disease progression blood draws for concurrent evaluation of CTCs and cfDNA (clinical trial registration ID, NCT00997334).

Materials and Methods

Study development

The primary objective of this open-label, nonrandomized phase II study was to prospectively assess the frequency of different genetic mechanisms of acquired resistance in patients with EGFR-mutant NSCLC treated with erlotinib until disease progression. Exploratory endpoints of this study included baseline assessment of CTCs and cfDNA in patients at study entry along with serial evaluation of both CTCs and cfDNA throughout treatment and at disease progression. The goal accrual for this study was determined based upon the primary endpoint. Specifically, a sample size of 60 patients was identified based upon achieving a goal of 41 evaluable patients with sufficient tissue available for mutation analysis from rebiopsy performed at the time of disease progression. A cohort of 41 patients with adequate rebiopsy allows for an 80% confidence interval (CI) of ±10% for an outcome (acquired T790M mutation) with an expected proportion of 50%. For the exploratory analyses included in this study, this represents a reasonable estimate of the true incidence of this genetic mechanism of secondary resistance. The protocol was approved by the Institutional Review Board of the Dana-Farber/ Harvard Cancer Center (Boston, MA; DF/HCC). Patients were enrolled at two DF/HCC institutions, the Dana-Farber Cancer Institute and the Beth Israel Deaconess Medical Center (Boston, MA). All patients provided written informed consent prior to initiating erlotinib therapy.

Patients

Eligible patients had advanced stage NSCLC with a sensitizing EGFR mutation identified through testing in a CLIA-approved laboratory and no prior history of treatment with EGFR-targeted agents. Patients could have had up to 1 prior line of non-EGFR-directed systemic therapy. Patients were required to be at least 18 years of age with an Eastern Cooperative Oncology Group performance status of 0 to 1. Exclusion criteria included untreated or unstable central nervous system (CNS) disease. A more detailed list of inclusion and exclusion criteria can be found in Supplementary Table S1. Patient records and baseline screening exams were also evaluated to identify the number of metastatic sites at study entry. Metastatic sites were defined as pleura, extrathoracic lymph nodes, bone, skin, omentum, CNS, and any visceral organ. Multiple nodules within the lung parenchyma or intrathoracic lymph nodes were not counted as separate metastatic sites. In addition, if a lesion had been irradiated prior to study entry, this site was not counted (26).

Study design

Eligible patients provided written informed consent and underwent baseline evaluation, including a baseline blood draw for assessment of CTCs and cfDNA, radiologic staging studies including CT scans of the chest and abdomen (pelvis if known disease in this region) and a brain MRI, and prospective assessment of demographic and tumor variables. Blood for cfDNA was collected in a single 10-mL heparin-containing Vacutainer tube, and blood for CTC analysis was collected in CellSave tubes per manufacturer’s instructions. Enrolled patients were reevaluated at the completion of each 28-day cycle for a repeat history, physical examination, and standard laboratory testing. After every 2 cycles, restaging chest and abdominal CT scans were performed along with serial blood draws for CTC and cfDNA analysis. Treatment was continued until disease progression as determined by clinician assessment of restaging scans and clinical assessment. Treatment could also be discontinued by patient withdrawal of consent. A blood draw for CTC and cfDNA analysis was performed at progression as defined above.
Patients were treated with 150 mg of erlotinib by mouth daily. Up to two dose reductions of 50 mg per reduction were permitted for treatment-related toxicities of grade 3 or higher or for intolerable grade 1–2 toxicities as defined by CTCAE criteria. Treatment compliance was monitored through a patient pill diary and pill counts at the end of each treatment cycle. Patients received full supportive care as clinically indicated for the duration of the study. At disease progression as defined above, prospectively consented patients were asked to undergo a repeat tumor biopsy. Tumor tissue underwent routine anatomic pathology review as well as evaluation for the T790M resistance mutation and, if possible, MET amplification. Testing options for T790M evolved during the course of this study and included laboratory single-gene PCR, multiplex allelic sequencing, and targeted next-generation sequencing (27, 28). MET amplification was evaluated by FISH at the Center for Advanced Molecular Diagnostics of the Brigham and Women’s Hospital Pathology Department (Boston, MA) and by next-generation sequencing (27). The consort diagram for this study is referenced in Supplementary Fig. S1 (30).

The cut-off date for data analysis was May 15, 2015.

CTC collection and analysis

After collection, samples for CTC analysis were processed with the CellTracks AutoPrep System using the CellSave Profile Kit (Veridex LLC) according to the manufacturer’s instructions. Cells were then prepared for both immunofluorescence (IF) and FISH analysis.

IF and FISH analyses were performed as described in detail in Supplemental Materials. Following IF slide preparation, a Bioview microscope at 400× magnification was used for microscopy evaluation. CTCs were counted as those cells with round to oval morphology, a visible nucleus (DAPI positive), positive staining for cytokeratin, and negative staining for CD45. Results of cell enumeration were expressed as the number of CTCs per 7.5 mL of blood. A sample was considered positive if a single cell meeting these criteria was identified. Following FISH slide preparation, a Bioview microscope at 600× magnification was used for microscopy evaluation of MET, EGFR, and CEP7 probes. Overdenatured signals were not included in evaluation. When a cell had 4 or more FISH signals from any FISH probes, a cell was classified as a CTC. When the ratio of the MET FISH signal to CEP7 FISH signal was greater than 2 per cell, the cell was defined as a MET-amplified cell. A sample was identified as MET amplified if any CTCs with MET amplification were identified. In the case of overlapping cells, all positive signals observed in the nucleus were counted and divided by the number of overlapping cells. Results of cell enumeration are expressed as the number of FISH-positive cells per 7.5 mL of blood. The cutoff for high level of CTCs was chosen as 3 or more cells per 7.5 mL of blood based upon the median of CTCs identified in those samples with detectable cells. Neither quartile assessment nor recursive partitioning identified a clear cut-off point for high versus low numbers of detectable CTCs and thus the median was used for data analysis.

cdDNA and droplet digital PCR of EGFR ex19del, L858R and T790M

After collection of patient blood samples as described, plasma was isolated by centrifugation at 1,200 × g for 10 minutes after which the supernatant was further cleared at 3,000 × g for an additional 10 minutes. Plasma was then stored in cryostat tubes at −80°C. cdDNA was isolated using the QIAmp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer’s protocol. Droplet digital PCR (ddPCR) of EGFR was performed as described previously (31), with primer and probe sequences noted in Supplementary Table S2. Analysis of the ddPCR data was performed with QuantaSoft analysis software (Bio-Rad) that accompanied the droplet reader. Notably, samples from 13 of the patients enrolled in this study were also utilized to validate the technical aspects of our ddPCR assay (31). cdDNA was considered detectable down to a level of 1 mutant copy/mL. A high level of cdDNA was defined as 55 or more copies/mL based upon quartiles, as this was found to be the 25th percentile value among those patients with detectable levels. Recursive partitioning was also used to identify an optimum cut-off point with an identified value of 52 copies/mL. As these two values were so similar, the initial value of 55 copies/mL based upon the 25th percentile was used for data analysis.

Statistical analysis

Goal accrual for this study was 41 patients with evaluable tissue from repeat biopsy at the time of disease progression. Assuming an underlying rate of 50% for the T790M mutation, the width of the two-sided 80% exact binomial CI for frequency of T790M mutation was estimated at 22%. After adjusting for an expected number of patients who would not undergo repeat biopsy, it was estimated that a total sample size of 60 patients would be required. Progression-free survival (PFS) was defined as the time in months from the date of erlotinib treatment initiation to the date of disease progression as identified by clinician assessment of restaging scans or death from any cause, whichever occurred first. Patients alive and progression free at the time of data analysis have been censored at their last known follow-up date up to a data cutoff of May 15, 2015. The Kaplan–Meier method was used to estimate event–time distributions and compared using the log-rank test. Cox proportional hazards models were fitted to estimate HRs. The Jonckheere–Terpstra test was used to test for associations between the number of metastatic sites and rate of high cdDNA and CTCs, respectively. All P values are two sided, and no adjustments have been made for multiple comparisons.

Results

Patient and tumor characteristics

Between February 2010 and January 2015, 60 patients were enrolled on study. The enrolled cohort had a median age of 62.5 years (range of 34–90), with 44 female patients (73%) and 34 never-smokers (57%). Three eligible patients enrolled on study with CNS metastases (5%; Supplementary Table S3). The median PFS for patients on study was 11.1 months. At the time of data cutoff, 48 of 60 patients had come off study, 44 for disease progression, and 4 for other events. (Supplementary Fig. S1; ref. 30).

Identification and analysis of CTCs and cdDNA at study baseline and disease progression

Forty-one of the 44 patients who came off study for progressive disease had paired CTC and cdDNA blood draws at study entry, whereas 36 patients had paired CTC and cdDNA blood draws at progression (Fig. 1). At disease progression, 36 of 44 patients had both CTC and cdDNA blood draws, 2 patients had only CTC blood draws, and 3 patients had only
cfDNA blood draws. (Fig. 1). CTCs were enumerated exclusively in baseline samples, with \textit{MET} FISH analysis performed in subsequent samples. \textit{EGFR} genotyping for L858R, exon 19 deletion, and T790M was performed in all samples collected for cfDNA analysis. Patients with other \textit{EGFR} genotypes were enrolled on study (Supplementary Table S3), but these rare genotypes were not evaluated by ddPCR.

At study baseline (Fig. 2A, left), of the 41 patients with paired CTC and cfDNA analysis, the most common finding was no detectable CTCs or cfDNA (n = 15, 37%). Nine samples (22%) had detectable CTCs but no detectable cfDNA, 11 samples (27%) had detectable cfDNA but no detectable CTCs, and 6 samples (15%) had detectable CTCs and cfDNA. Of the 17 samples with detectable cfDNA, an L858R mutation was identified in 6 and an exon 19 deletion in 12. Detection of \textit{EGFR} exon 19 deletion and L858R was 100% concordant between cfDNA analysis and tissue biopsy analysis. Shown for comparison are the outcomes of \textit{EGFR} mutation testing in tissue biopsies at study baseline (Fig. 2A, right). In baseline samples, the median number of CTCs and cfDNA was 0 (range 0–1,146). In baseline samples, the median number of mutant copies of \textit{EGFR} identified by ddPCR was 0 (range 0–1,649).

Paired CTC and cfDNA analysis was available at the time of disease progression in 36 of 44 patients (Fig. 2B, left). Among this cohort, the most common finding was the absence of detectable CTCs and cfDNA (n = 15, 42%). Twelve samples (33%) had detectable CTCs but no detectable cfDNA, 3 samples (8%) had detectable cfDNA but not detectable CTCs, and 6 samples (15%) had detectable CTCs and cfDNA. cfDNA analysis at progression identified 9 T790M samples. Also shown is a direct comparison of CTC and cfDNA analysis to the outcomes of repeat tissue biopsy performed in 35 of 44 patients at progression (Fig. 2B, right).

Correlation between metastatic sites at diagnosis and presence of baseline CTCs or cfDNA

Baseline samples were obtained for CTC or cfDNA analysis in 41 patients. CTC analysis (Fig. 3A) or baseline cfDNA copy number of mutant \textit{EGFR} (Fig. 3B) was evaluated using the Jonckheere–Terpstra test. Association between the number of metastatic sites at diagnosis and presence of baseline CTCs (Fig. 3A) or baseline cfDNA copy number of mutant \textit{EGFR} (Fig. 3B) demonstrated an association with high cfDNA (P = 0.02), but not with CTCs (P = 0.38).

Serial analysis of CTCs and cfDNA throughout study enrollment

Thirty-six of 44 patients with disease progression had a baseline blood draw, serial blood draws on study, and a progression blood draw for paired analysis of both CTCs and
cDNA. Analysis of paired CTC and cfDNA results revealed four major patterns as demonstrated by representative panels in Fig. 4. In 6 samples, both CTCs and cfDNA were detected near study baseline, followed by disappearance of both CTCs and cfDNA during patient clinical response to erlotinib and resumed detection of both CTCs and cfDNA around disease progression (Fig. 4A). In 9 samples, CTCs and/or cfDNA were detected only around disease progression (Fig. 4B). In 7 samples, neither CTCs nor cfDNA were detected at any time during treatment (Fig. 4C). Finally, in the remaining 14 samples, there was no clear correlation between cfDNA and CTCs at baseline, during study, or at disease progression (Fig. 4D and E). Notably, in some cases in which CTCs were identified intermittently during prolonged clinical response to erlotinib, further evaluation of these CD45+/cytokeratin+ cells revealed normal ploidy on chromosome 7 (EGFR and MET), suggesting that CD45- staining as identified through IF may not always represent tumor cells (Fig. 4F). An example of a CD45-/cytokeratin+ cell with polysomy by FISH is shown in Supplementary Fig. S2.

Baseline genomic mutations and mechanisms of acquired resistance identified in analysis of CTCs and cfDNA as compared with tissue biopsies

A comprehensive serial assessment of each of the 44 patients who came off study for progressive disease is shown in Fig. 5. The left panel shows all cases with T790M identified on repeat tissue biopsy. Overall, 35 patients with progressive disease underwent repeat tumor biopsy, and analysis identified a T790M mutation in 23 of 35 (66%), a small cell transformation (SCLC) on routine anatomic pathology review in 3 of 35 (9%), a MET amplification in 1 of 35 (3%), adequate biopsy tissue but unknown mechanism of resistance in 4 of 35 (11%), and insufficient tissue for analysis in 4 of 35 (11%). Notably, 9 patients with progressive disease did not undergo repeat biopsy because of patient refusal (3), patient rapid demise and death from disease progression (2), lack of safe lesion to biopsy (dome of liver; 1), clinical need for urgent therapy (2), and identification of baseline T790M mutation (1). The patient with the baseline T790M mutation did not respond to erlotinib and had radiographic disease progression at the first scheduled restaging scan.
In contrast, only 3 patients did not have a blood draw for evaluation of CTCs and/or cfDNA at disease progression. A sample for cfDNA analysis was obtained in 39 of 44 (89%) patients, and cfDNA was detected in 9 of 39 samples (23%). A T790M mutation was identified in 7 of 39 (18%) samples, all of which were also found to harbor a T790M mutation on tissue biopsy. In addition, a circulating T790M mutation was identified in two additional samples, both patients in whom no tissue biopsy was performed because of the lack of a safe lesion to biopsy or the need for more urgent palliative treatment of bony lesions.

A sample for CTC analysis was obtained in an overlapping cohort of 38 of 44 patients (89%), with detectable CTCs identified in 18 of 38 (47%) samples. MET amplification was detected in CTCs in 3 of 39 samples (8%). The first patient had a T790M mutation identified by tissue biopsy and cfDNA at disease progression, although MET amplification was not identified on FISH analysis of the tissue biopsy. The second patient was found to have a SCLC transformation on pathology review of the tissue biopsy at disease progression and did not have sufficient tissue remaining for MET copy number assessed by FISH. As previously noted, 3 of 35 patients who underwent repeat tissue biopsy were found to have an SCLC transformation on routine anatomic pathology review. The patient with SCLC transformation and concurrent MET amplification identified in CTCs had the shortest overall survival (OS) from time of progression on erlotinib compared with the other two patients with SCLC transformation without identification of MET amplification (7.2 months compared with 11.8 months and 17.9 months). The third patient in whom MET amplification was identified in CTCs at disease progression was not found to have T790M in evaluation of repeat biopsy tissue or cfDNA but was found to have MET amplification in FISH analysis of the repeat biopsy tissue.

In patients with MET amplification identified in CTCs, MET-amplified cells were only identified at the disease progression biopsy and were not found during serial assessment on study. In the 10 samples in which an EGFR T790M mutation was identified in cfDNA, a positive cfDNA result pre-dated a clinical and/or radiographic assessment of disease progression in 6 of 9 patients.

**PFS in patients with high or low level CTCs or cfDNA at baseline**

PFS on erlotinib was determined for all 60 patients enrolled on study based upon high or low levels of CTCs or cfDNA (Fig. 6).

The cutoff for a high level of CTCs was defined as ≥3 CTCs per 7.5 mL. Patients without detectable CTCs were also included in the low CTC category. High levels of cfDNA were considered to be ≥55 EGRF mutation copies/mL compared with low cfDNA with <55 EGRF-mutant copies/mL. Patients without detectable cfDNA were also included in the low cfDNA category. The median PFS for patients with high baseline CTCs (n = 15) was 11.0 months (95% CI, 6.0–NA) versus 10.9 months (95% CI, 6.8–16.7) for patients with low baseline CTCs (n = 41; P = 0.88; Fig. 6A). In contrast, the median PFS for patients with high baseline cfDNA (n = 18) was 9.3 months (95% CI, 6.3–14.8) versus 14.0 months (95% CI, 9.2–20.1) for patients with low baseline cfDNA (n = 41; P = 0.08; Fig. 6B).

**Discussion**

In this prospective phase II trial, we were able to directly compare tissue biopsy and noninvasive biopsy at study entry and at disease progression while also evaluating the results of serial cfDNA and CTCs for disease monitoring throughout treatment with erlotinib. Our findings support previous work demonstrating the relative clinical ease of noninvasive biopsy (1) and also identify several areas in which evaluation of CTCs and cfDNA may augment existing strategies for evaluating acquired resistance in NSCLC patients treated with targeted therapies. This approach may be particularly relevant for patients in whom tumor tissue may not be easily or safely obtained.

First, the majority of study patients who progressed on erlotinib were able to undergo a blood draw for evaluation of CTCs and/or cfDNA at study progression (41/44, 93%). As the therapeutic landscape continues to evolve for NSCLC patients with a potentially targetable genomic alteration, a list that continues to grow and includes not only EGFR mutations and ALK rearrangements but also ROS1 rearrangements (5), MET amplification (32), and BRAF mutations (33), among others, the importance of repeat biopsy for genomic evaluation of emerging acquired resistance mechanisms is clear. A direct comparison of invasive and noninvasive biopsy at disease progression demonstrates that among the 44 patients with disease progression, 43 of 44 (98%) were able to have either a tissue biopsy (35, 80%) or a noninvasive liquid biopsy with CTC and/or cfDNA analysis (41, 93%), with 34 patients successfully having both a tissue biopsy and noninvasive analysis (77%). Interestingly, of the 9 patients who did not
Figure 4.
Patterns of serial CTC and cfDNA analysis. A–E, The major patterns of serial CTC and cfDNA evaluation of the 44 patients who progressed while on study. CK, cytokeratin; Ex19 del, exon 19 deletion. F, Top, IF of representative CTCs from the time point indicated with a black arrow in Fig. 3E. Blue cells are counted as CTCs based on positive cytokeratin staining (yellow) and negative CD45 staining (red); bottom, EGFR and MET FISH analysis on the same slides shown on top.
undergo repeat tissue biopsy at the time of disease progression, 8 did have a noninvasive biopsy which, in some cases, identified clinically relevant information. A circulating T790M mutation was identified in cfDNA analysis from 2 of these patients: an individual who did not have a metastatic lesion amenable for conventional tissue biopsy because of safety concerns and an individual with an urgent need for palliative radiotherapy. In this study, there were no patients in whom a T790M mutation was identified in cfDNA but not in tumor biopsy. However, recent reports do indicate that tumor biopsy can miss a T790M mutation that is ultimately identified in a liquid biopsy (19). Together, the findings in our study suggest that noninvasive biopsies may be a clinically actionable option for patients unable to undergo a more conventional tissue biopsy and may also represent a possible strategy to ensure that intratumoral heterogeneity does not cause an actionable mutation to be missed on conventional tissue analysis (34).

However, it is important to note that while noninvasive biopsy methods did demonstrate potentially actionable mutations in 2 patients without available tissue biopsy, there were limitations to the extent of an evaluation coming exclusively from a blood draw because not all tumors shed cfDNA and/or CTCs. Indeed, the variable patterns of CTC shed and circulating cfDNA as shown in Fig. 4 further underscore the potential

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Figure 5.
Genomic findings at baseline, throughout study, and at disease progression in tumor tissue, CTCs, and cfDNA. Serial depiction of genomic findings at study baseline, throughout study, and at disease progression for each patient who progressed while on study. Included here are data from tissue biopsy at study entry and progression, along with the results of serial CTC and cfDNA analysis.
apy regimens (35), thus identifying this transition is an important step in the evaluation and treatment of patients with progressive disease on EGFR TKIs. Of note, this study spanned a critical time in the ongoing technological development of CTC and cfDNA collection and analysis, and improved methodology will continue to increase the sensitivity and specificity of noninvasive biopsies moving forward (31, 36). Work from our own group has demonstrated improved detection of cfDNA compared with the results shown here, likely a result of modifications made to blood collection protocols to avoid heparinized collection tubes because of potential interference with ddPCR (31, 37). More comprehensive sequencing methods, including next-generation sequencing capable of detecting MET amplification and whole-exome sequencing, have now been reported based upon cfDNA analyses (37, 38). Moreover, a recent report demonstrates that sequencing in CTCs can identify the T790M mutation (19). However, as the comprehensive data from this study make clear, invasive and noninvasive biopsy methods have areas of overlap as well as distinct advantages or disadvantages in the evaluation of patients with disease progression on targeted therapies; thus, tissue biopsy and noninvasive analysis will likely continue to play a role in the overall evaluation and treatment of patients with NSCLC.

Moving forward, the results of this study suggest clinical benefit to continued integration of conventional tissue biopsy and noninvasive biopsy methods. The identification of MET amplification in CTCs isolated from a patient with a concurrent T790M mutation and from a patient with SCLC transformation emphasizes that acquired resistance may often be more nuanced than a dependence on a single dominant signaling pathway. This rationale underlies the ongoing TATTON study (clinical trial registration ID, NCT02143466) combining the third-generation EGFR T790M inhibitor osimertinib with either a MET or MEK inhibitor. The findings from this study support testing using a combination of invasive and noninvasive methods for assessing evolving changes in patients with oncogenic drivers undergoing treatment with targeted agents. Overall, a more comprehensive approach to the analysis of patients with acquired resistance to EGFR TKIs will allow the best utilization of both approved therapies and investigational agents and combinations for these patients.

Finally, a robust literature continues to evaluate CTCs as a prognostic biomarker as well as a means of monitoring metastatic disease in breast cancer (39, 40), but the data for prognostic use of CTCs in NSCLC have not been as clear thus far. In this study, we show that actionable genetic information can be obtained from both CTCs and cfDNA, consistent with a recent report demonstrating identification of the EGFRT790M mutation in evaluation of CTCs and cfDNA (19). However, in this cohort, evaluation of high versus low detection of cfDNA appears to be a potentially more effective prognostic biomarker than evaluation of high versus low detection of CTCs at the time of disease diagnosis (Fig. 6). Whether this finding is a result of biological differences in shedding between cfDNA and CTCs or a result of the analytic methods used in this study is currently unknown. As reported here, CTCs are detected in low single digits, while the measurement by ddPCR of cfDNA exhibits a large dynamic range and may thus reflect a more granular representation of the tumor. Underlying variation in tumor biology based upon anatomic site of origin may ultimately help direct the significance of a tumor-shedding cfDNA or CTCs (or both). Finally, data from our study suggest that some cells identified as CTCs because characteristic variation that can be seen in noninvasive assays. In this study cohort, the most common mechanism of acquired resistance to first-line EGFR TKIs, the T790M mutation found in approximately half of patients with acquired resistance (9), could not be evaluated in a blood sample in 34 of the 44 patients with progression (77%) because of either the failure to collect a noninvasive blood draw (n = 5) or the fact that cfDNA was simply not detected in a majority of the plasma samples (n = 29). Similarly, although published rates of MET amplification as a mechanism of acquired resistance to EGFR TKIs vary (8, 32), amplification in CTCs could only be assessed in 18 of 44 (41%) patients in this study. Finally, noninvasive analysis as utilized here was unable to identify SCLC transformation. These patients can respond to conventional SCLC chemotherapy regimens (35), thus identifying this transition is an important step in the evaluation and treatment of patients with progressive disease on EGFR TKIs. Of note, this study spanned a critical time in the ongoing technological development of CTC and cfDNA collection and analysis, and improved methodology will continue to increase the sensitivity and specificity of noninvasive biopsies moving forward (31, 36). Work from our own group has demonstrated improved detection of cfDNA compared with the results shown here, likely a result of modifications made to blood collection protocols to avoid heparinized collection tubes because of potential interference with ddPCR (31, 37). More comprehensive sequencing methods, including next-generation sequencing capable of detecting MET amplification and whole-exome sequencing, have now been reported based upon cfDNA analyses (37, 38). Moreover, a recent report demonstrates that sequencing in CTCs can identify the T790M mutation (19). However, as the comprehensive data from this study make clear, invasive and noninvasive biopsy methods have areas of overlap as well as distinct advantages or disadvantages in the evaluation of patients with disease progression on targeted therapies; thus, tissue biopsy and noninvasive analysis will likely continue to play a role in the overall evaluation and treatment of patients with NSCLC.

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cytokeratin and CD45 staining on IF may not all be tumor cells because of normal diploid expression of EGFR and MET FISH markers instead of the amplification and/or polysomy that is frequently observed in NSCLC tumors with mutations in EGFR (41). Undoubtedly, the technology for evaluation of CTCs and cfDNA will continue to develop, but in an intensely genetically driven disease, such as NSCLC; cfDNA analysis or sequencing-based evaluation of CTCs may ultimately become a more clinically meaningful tool than enumeration of CTCs. Overall, incorporating noninvasive methods of blood-based genomic analyses into current clinical and research algorithms for patients with NSCLC can extend our ability to monitor disease, investigate mechanisms of acquired resistance to targeted therapies, and best leverage available treatment options to improve patient outcomes.

Disclosure of Potential Conflicts of Interest

A. J. Redig is a consultant/advisory board member for Medtronic. G. R. Oxnard is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Genentech, and Inviva. B. E. Johnson has ownership interest (including patents) in and is a consultant/advisory board member for K2W Group. D. B. Costa has received speakers bureau honoraria from Pfizer and is a consultant/advisory board member for AstraZeneca and Boehringer Ingelheim. D. M. Jackman is a consultant/advisory board member for Genentech. P. A. J. Anne reports receiving commercial research grants from AstraZeneca and Astellas; has ownership interest (including patents) in Gatekeeper Pharmaceuticals; is a consultant/advisory board member for Ariad Pharmaceuticals, AstraZeneca, Boehringer Ingelheim, Chugai Pharmaceuticals, and MetrImack Pharmaceuticals; and has provided expert testimony for LabCorp. No potential conflicts of interest were disclosed by the authors.

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Grant Support

This study was supported by NCI grants T32CA0909172 (supporting A. J. Redig), 5R01CA144665 (to B. E. Johnson, P. A. J. Anne, and G. R. Oxnard); and 5R01CA135257 (to G. R. Oxnard and P. A. Johnson), Kaplan Research Fund for Thoracic Oncology (to S. E. Dahlberg), and Expect Miracles Foundation (to C. P. Paweletz). Funding for this study was also provided by Genentech. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 13, 2016; revised May 21, 2016; accepted May 23, 2016; published online First June 8, 2016.

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6019

Published OnlineFirst June 8, 2016; DOI: 10.1158/1078-0432.CCR-16-0909

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A Prospective Evaluation of Circulating Tumor Cells and Cell-Free DNA in *EGFR*-Mutant Non–Small Cell Lung Cancer Patients Treated with Erlotinib on a Phase II Trial


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