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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Evaluation of Circulating Tumor Cells and Circulating Tumor DNA in Non–Small Cell Lung Cancer: Association with Clinical Endpoints in a Phase II Clinical Trial of Pertuzumab and Erlotinib

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Abstract

Purpose: Elevated levels or increases in circulating tumor cells (CTC) portend poor prognosis in patients with epithelial cancers. Less is known about CTCs as surrogate endpoints or their use for predictive biomarker evaluation. This study investigated the utility of CTC enumeration and characterization using the CellSearch platform, as well as mutation detection in circulating tumor DNA (ctDNA), in patients with advanced non–small cell lung cancer (NSCLC).

Experimental Design: Forty-one patients were enrolled in a single-arm phase II clinical trial of erlotinib and pertuzumab. Peripheral blood was analyzed for CTC enumeration, EGFR expression in CTCs, and detection of oncogenic mutations in CTCs and ctDNA. Changes in CTC levels were correlated with 2[18F]fluoro-2-deoxy-D-glucose–positron emission tomographic (FDG-PET) and computed tomographic (CT) imaging and survival endpoints.

Results: CTCs were detected ($\geq$1 CTC) at baseline in 78% of patients. Greater sensitivity for mutation detection was observed in ctDNA than in CTCs and detected mutations were strongly concordant with mutation status in matched tumor. Higher baseline CTC counts were associated with response to treatment by Response Evaluation Criteria in Solid Tumors (RECIST, $P = 0.009$) and decreased CTC counts upon treatment were associated with FDG-PET and RECIST response ($P = 0.014$ and $P = 0.019$) and longer progression-free survival ($P = 0.050$).

Conclusion: These data provide evidence of a correlation between decreases in CTC counts and radiographic response by either FDG-PET or RECIST in patients with advanced NSCLC. These findings require prospective validation but suggest a potential role for using CTC decreases as an early indication of response to therapy and ctDNA for real-time assessment of mutation status from blood. *Clin Cancer Res; 18(8); 2391–401. ©2012 AACR.*

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, accounting for more than 1.2 million deaths annually (1). Approximately 85% of lung cancers can histologically be defined as non–small cell lung cancer (NSCLC). The majority of patients with NSCLC are diagnosed with inoperable, metastatic disease. In these patients, 5-year survival rates are around 2% (1). Understanding the molecular basis and oncogenic drivers of lung cancer is crucial to the development of novel targeted therapies. The signaling pathway regulated by the EGF receptor (EGFR) is an important axis in the pathogenesis of lung cancer, and EGFR mutations are found in 10% to 20% of lung adenocarcinomas (2, 3). Erlotinib (TARCEVA) is designed to inhibit the tyrosine kinase activity of EGFR, and erlotinib treatment has been shown to prolong survival of patients with advanced NSCLC who have failed at least one round of prior chemotherapy (4). Erlotinib shows particularly dramatic antitumor activity in patients whose tumors harbor activating EGFR mutations (2). Pertuzumab is a humanized monoclonal antibody directed against HER2 that is designed to inhibit dimerization of HER2 with other HER family receptors including EGFR, HER3, and HER4 (5–7). Previous phase II studies in NSCLC have shown signs of

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Translational Relevance

Advanced non–small cell lung cancer (NSCLC) is the leading cause of cancer death worldwide and is a significant unmet medical need. More facile measures of disease progression and access to tumor material for diagnostic evaluation could significantly aid in the clinical management of this disease. We show in this study that evaluation of circulating biomarkers, specifically circulating tumor cells (CTC) and circulating tumor DNA (ctDNA), can provide information about the molecular characteristics of a patient's tumor from a noninvasive blood draw. Moreover, we provide, to our knowledge, the first evidence that decreases in CTC numbers during treatment of patients with advanced NSCLC with targeted therapies are associated with clinical responses measured by 2[18F]fluoro-2-deoxy-D-glucose–positron emission tomographic (FDG-PET) and computed tomographic imaging.

pharmacodynamic activity in response to single-agent pertuzumab therapy in patients with advanced or recurrent NSCLC, though no partial or complete responses were observed (8, 9). Pertuzumab and erlotinib inhibit overlapping but distinct aspects of HER family signaling, and other studies have suggested that development of acquired resistance to EGFR inhibitors is associated with upregulation and increased dependency on other HER family members such as HER3 (10), providing a strong rationale for studying the safety and efficacy of the combination of these agents in advanced NSCLC.

There are a number of issues that complicate successful development of new therapies or therapeutic combinations in lung cancer. One issue is the lack of surrogate markers of antitumor activity that could provide an early indication of response to therapy and help guide clinical decision making (11). A second issue is the inherent difficulty in obtaining representative lung tumor specimens to explore and validate predictive tissue biomarkers of targeted therapies (12). It is even more challenging to obtain serial biopsies from the same patient, though such samples can be essential to understand acquired resistance mechanisms and identify appropriate therapies upon progression (13). Circulating tumor cell (CTC) enumeration and characterization have potential to address some of these issues. Specifically, monitoring CTC numbers in patients while on treatment could have application as a surrogate endpoint of antitumor activity. Studies in breast, colorectal, and prostate cancer have shown the prognostic significance of CTCs and further suggested that changes in CTC numbers may be predictive of response in patients receiving standard therapy (14–19). In addition to the potential prognostic and predictive potential of CTC enumeration, molecular characterization of CTCs holds promise in the evaluation of predictive biomarkers for targeted therapies (20–25). CTCs could potentially provide a real-time snapshot of the molecular makeup of a patient's cancer, essentially serving as an easily accessed "liquid biopsy." In addition, as these cells may be involved in the establishment of sites of metastasis, such analyses could conceivably be more relevant than those conducted on initial tumor samples.

In addition to known prognostic significance in breast, colorectal, and prostate cancer, CTC enumeration has recently been shown to have prognostic significance in patients with metastatic NSCLC treated with one cycle of standard chemotherapy (26). Specifically, patients with less than 5 CTC at baseline showed both progression-free survival (PFS) and overall survival (OS) benefit than patients with 5 or more CTCs at baseline, with baseline CTC numbers being the strongest predictor of OS in multivariate analysis. In addition, this study found that decreases in CTC numbers upon therapeutic intervention were predictive of both PFS and OS benefit (26), suggesting that on-treatment evaluation of CTC changes from baseline may be predictive of patient response to standard therapy in NSCLC.

In this study, we evaluated CTC numbers and changes over the course of treatment in the context of a global, multicenter phase II study of erlotinib and pertuzumab in patients with advanced NSCLC (27). The primary outcome measures for this study were 2[18F]fluoro-2-deoxy-D-glucose–positron emission tomographic (FDG-PET) response at day 56 and determination of EGFR mutation status in tumor tissue. PFS, OS, and safety were secondary objectives in this study. In addition, an exploratory objective of the study was to compare CTC numbers and changes over the course of treatment and plasma tumor DNA analytes with the primary and secondary outcome measures. Several limitations of study design should be considered when interpreting the results. First, the predictive or prognostic nature of biomarker correlations with survival endpoints observed cannot be distinguished because of the single-arm nature of the trial. Second, longitudinal biomarker sampling in this study was limited to patients who remained on study at the time points of assessment (days 14, 28, and 56). Because the sampling may be, therefore, enriched for responders, the magnitude of the biomarker changes observed in this study may be larger than if sampled in all patients originally enrolled in study. Finally, biomarker analyses were retrospective and exploratory and hence will require prospective validation.

The specific, CTC-related exploratory goals of this study were as follows: (i) to evaluate the sensitivity of detecting CTCs in advanced lung cancer and the feasibility of CTC evaluation in a phase II trial setting and (ii) to evaluate whether CTC counts are correlated with radiographic response and patient survival and (iii) to evaluate whether candidate predictive biomarkers can be assessed through molecular and cell–based assays on captured CTCs.

Patients and Methods

Study design

The study was a phase II, open-label, single-arm, single-stage, multicenter trial in patients with relapsed NSCLC.

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Enrollment in the study was not based on tumor EGFR or HER2 status. Details of the study, including efficacy and safety endpoints, have been reported elsewhere (27) and will be described in depth in a future publication. This study enrolled 41 relapsed or patients with refractory NSCLC in 9 study centers in the United States and Australia. Before beginning dosing in this study, and after having met all other eligibility criteria, patients underwent baseline imaging with FDG-PET and computed tomographic (CT) scans of the chest, abdomen, and pelvis. Only patients who had at least one concordantly evaluable lesion on both CT and FDG-PET scans were eligible for the study. FDG-PET imaging was carried out at baseline and at days 14, 28, and 56, whereas CT imaging was carried out at baseline and days 56 and 105 for patients on study. Patients received a first pertuzumab infusion (840-mg loading dose), followed by daily doses of erlotinib (150 mg *per os*), with subsequent pertuzumab infusions (420 mg) every 3 weeks. Availability of patient tumor sample was an eligibility requirement. Peripheral blood was collected by optional consent for CTC analysis at baseline (two 10-mL tubes) and at days 14, 28, and 56 (one 10-mL tube) if patient stayed on study. In addition, plasma (one 10-mL blood tube) was collected also by optional consent both at baseline and the same on-study time points. Data were collected for age, ethnicity, histologic subtype, smoking status, Eastern Cooperative Oncology Group (ECOG) performance status, survival, and response (RECISTv1.0).

CTC enumeration and EGFR expression analysis

CTC enumeration on the CellSearch platform was carried out according to the manufacturer's training and protocol (Veridex), and all samples were run at Genentech. Blood samples were collected in CellSave (Veridex) preservative tubes, shipped at ambient temperature, and processed upon arrival. The sensitivity, accuracy, linearity, and reproducibility of the CellSearch system have been described previously (28, 29). EGFR expression on CTCs was evaluated on the open fluorescein isothiocyanate (FITC) channel on CellSearch with the EGFR CellSearch Tumor Phenotyping Reagent (Veridex). EGFR immunofluorescence scoring criteria were developed by analysis of tumor cell lines with varying expression levels recovered from "spike in" experiments, as described previously (20).

Mutation analysis

For mutation analysis, CTCs were captured by the RUO Profile Kit (Veridex) and DNA extracted as described previously (20). DNA was extracted from 2 mL of plasma by the Circulating Nucleic Acid kit (Qiagen) as per manufacturer's protocol. About 20 ng of the extracted DNA from the CTC preparation, plasma circulating tumor DNA (ctDNA), or 1 to 20 ng of the tumor DNA, was used in a preamplification reaction before mutation detection by TaqMan. The preamplification step is a multiplex gene-specific PCR reaction using primers that generate 100 to 120 basepair amplicons around the following mutation sites: exons 18, 19, 20, and 21 in EGFR including the T790M mutation, codons 12 and 13 in KRAS exon 2, codon 600 in BRAF exon 15, codons 542, 545, and 1,047 in PIK3CA exons 10 and 20, codons 12 and 61 in NRAS exons 2 and 3, and codon 17 in AKT1 exon 4. EGFR mutations were determined by DxS kits (Qiagen) as per manufacturer's protocol with minor modifications, such as the preamplification step for exons 18 to 21 and the use of a custom real-time quantitative TaqMan assay for the EGFR exon 20 wild-type sequence. KRAS mutations were determined by both in house TaqMan Assays and DxS kits (Qiagen). Mutations in PIK3CA, BRAF, NRAS, and AKT1 were detected by custom designed TaqMan genotyping assays (ABI). Primers and probes for these assays are listed in Supplementary Table S1.

PET imaging

The assessment of PET responses in this study was conducted by a central reading site and was based on the maximum uptake (SUV_{max}) of up to 5 regions of interest (ROI). ROIs were identified for each patient on pretreatment FDG-PET scans; those that corresponded to preselected CT Response Evaluation Criteria in Solid Tumors (RECIST) target lesions were selected for quantitative analysis. Determination of PET response was based on the definitions proposed by the EORTC (30). Specifically, the SUV_{max} of each ROI on the on-treatment scans was compared with its SUV_{max} on the corresponding pretreatment scan and the percentage of change was determined. When there was more than one ROI, the overall percentage of change in SUV_{max} was defined as the arithmetic mean of the percentage of changes in SUV_{max} for each of the ROIs ($mSUV_{max}$). An objective PET response was defined as a decrease of 20% or more in $mSUV_{max}$. They were further categorically defined as CR, patients with 100% decrease in $mSUV_{max}$; PR, patients with 20% or more decrease; PD, patients with 20% or more increase; and SD, patients with changes between PR and PD.

Statistical analyses

Statistical analyses were done with the analysis software Prism (GraphPad Software Inc). For correlation of CTC counts with PET response and PFS at the early time points, CTC data were pooled together for the day (D) 14 and D28 time points, with D28 data used if both time points were available. The D56 time point was used for CTC counts when comparing with the D56 CT scan response data, and response was determined as per central reading site assessment. PFS was determined by investigator assessment. For correlating treatment-associated changes in CTC counts with mutation status, CTC data were pooled together for the D14, D28, and D56 time points, with latest time point used if multiple time points were available. All available data were used for each analysis, though numbers vary in the analyses due either to samples not being collected for a given time point, or not being collected after patients left the study upon clinical progression.

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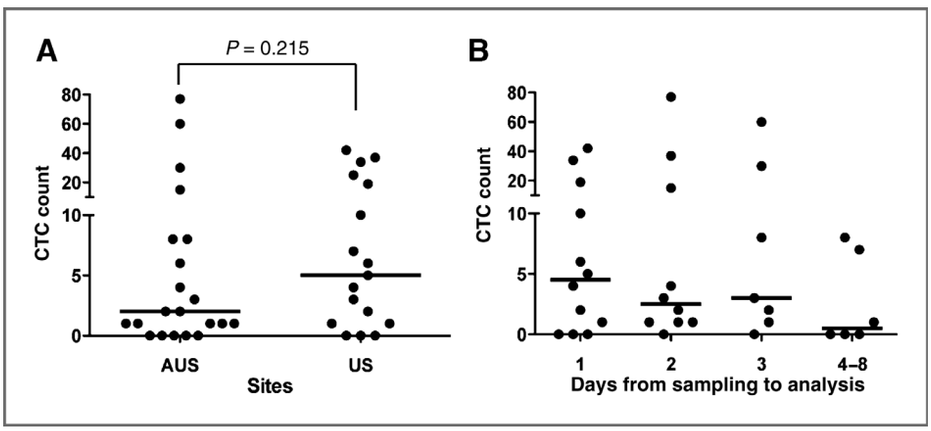

Figure 1. CTC counts in patient blood samples binned by (A) country of shipment or (B) by time from sampling to analysis. AUS, Australia; US, United States.

Results

CTC results

Forty out of the 41 patients in the study consented to the optional blood collection for evaluation of CTCs. Of the 97 blood draws across all time points, 11 were not evaluable for CTC enumeration due to technical issues. These issues included instrument failure ($n = 10$) and insufficient blood volume ($n = 1$). CTC were detected (CTC $\geq$ 1) in 76% of patients at baseline (28 of 37 patients with evaluable blood). Blood was shipped for centralized analysis from 9 sites, including 5 in Australia and 4 in the United States, with a range of 1 to 8 days from sampling to analysis. Shipping times averaged 2.9 days (range, 2–7 days) from Australian sites and 1.5 days (range, 1–8 days) from U.S. sites. Despite the slightly longer shipping times from Australian sites, median CTC counts were not significantly different between U.S. and Australian sites (U.S. sites median, 4.5; range, 0–37; AUS sites median, 2; range, 0–77; $P = 0.215$, Mann–Whitney U test, Fig. 1A). When samples were binned by shipping time, a slight trend was observed toward lower CTC counts in samples analyzed for 4 days or more postblood collection, but the difference was not statistically significant between time points ($P = 0.542$, Kruskal–Wallis test, Fig. 1B). Overall, these data are consistent with previous reports that CTC levels are maintained in CellSave tubes when samples are analyzed within 96 hours. A minority of samples (18%) missed the 96-hour recommended time frame for analysis from CellSave tubes. Whereas this study used a single centralized laboratory, a model with multiple regional laboratories may best ensure that all samples are analyzed within 96 hours when arriving from international sites.

Baseline CTC counts before treatment and association with patient characteristics and blood collection metrics are listed in Table 1. There was a statistically significant correlation between high baseline CTC counts and patient response to treatment by RECIST ($P = 0.009$). Trends were observed with high baseline CTC counts and older age of patient as well as EGFR or KRAS mutation status compared with wild-type but these did not reach statistical significance. These findings are discussed in more detail later.

EGFR expression in CTCs

EGFR expression was evaluated by immunofluorescence microscopy in CTCs from 26 patients (Fig. 2A and B). EGFR expression in CTCs varied widely across patients in the study, ranging from uniformly high expression (3+) to uniformly low expression (0). A subset of patients showed heterogeneity of EGFR levels in their CTCs, with both high and low expressing CTCs observed in samples from the same patient. Because EGFR protein expression could be affected by time elapsed from collection to analysis, we tested whether EGFR levels were associated with country of origin or shipping time but found no significant correlation. EGFR expression in CTCs was not associated with tumor EGFR mutation status ($P = 0.19$, Mann–Whitney U test) or other oncogenic mutations detected in these patients (Fig. 2A).

Mutation detection in CTCs and ctDNA

DNA was extracted from isolated CTCs and independently from ctDNA isolated from patient plasma. Assay development using spiked-in cell lines for mutation detection in CTCs on CellSearch was previously described (20). CTC samples for mutational analyses were only available at baseline, whereas ctDNA samples were available for baseline as well as on-treatment time points. DNA from both sources was used for mutational analysis with a 6-gene mutation panel consisting of assays for the most common alterations in EGFR, KRAS, BRAF, NRAS, AKT1, and PIK3CA. Because mutational analysis was conducted by quantitative PCR, we were also able to compare the strength of the mutant signal at baseline and with on-treatment time points. Only one EGFR mutation (exon 19 deletion) was detected in CTC-derived DNA from the 38 patient samples analyzed, despite the fact that 8 patients had confirmed tumor EGFR mutations in archival tissue and CTCs for mutation analysis (Supplementary Table S2). The same mutation was also detected both in the patient's matched plasma ctDNA sample (Supplementary Fig. S1) and archival tumor. Higher ΔC_t values at day 0 (D0) suggested that mutant EGFR signal was more abundant in ctDNA in comparison with DNA extracted from CTCs. Mutational

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Table 1. CTC counts before initiating treatment and association with clinical and blood collection characteristics

Characteristic	Points with CTC data	*P* value Mann–Whitney
Age, y		
<65	24	
≥65	13	
P		0.0556
Race		
Caucasian	33	
Asian	4	
P		0.5572
Histology		
Adenocarcinoma	24	
Squamous	9	
P		0.9837
Gender		
Male	21	
Female	16	0.8042
Smoking		
Yes	31	
No	6	
P		0.8026
Genotype		
EGFR mut	8	
EGFR WT	21	
KRAS mut	7	
KRAS WT	24	
EGFR WT, KRAS WT	14	
P value EGFR mut vs. WT		0.0941
P value KRAS mut vs. WT		0.1808
P value EGFR mut vs. WT for KRAS and EGFR		0.0621
P value KRAS mut vs. WT for KRAS and EGFR		0.0648
Tumor burden		
Sld at baseline (below median)	18	
Sld at baseline (above median)	19	
P		0.747
RECIST response		
Partial response	7	
Stable disease or progressive disease	12	
P		0.0091
ECOG		
0	9	
1	21	
2	7	
P		0.4813
Blood shipment site		
Australia	21	
US	16	
P		0.6309
Blood shipment time		
≤3 d	28	
>3 d	6	
P		0.3682

NOTE: *P* value is based on 2-tailed Mann–Whitney *U* test except for ECOG score which used a Kruskal–Wallis test for 3-way comparison.

Abbreviation: Sld, sum of the longest unidimensional diameter.

analyses of ctDNA showed decreased signal with the EGFR mutation assay and decreased ΔC_t values at day 56 compared with day 0, suggesting reduced mutational load upon treatment in this patient. This patient also showed a partial response by RECIST criteria and FDG-PET imaging at day 56 (Fig. 2D).

Plasma samples were available from a total of 25 patients and ctDNA samples were analyzed for the presence of mutations in EGFR, KRAS, PIK3CA, and BRAF. EGFR and KRAS mutations in ctDNA were mutually exclusive and were found in 4 and 5 patients, respectively. Mutations detected in ctDNA for EGFR and KRAS were completely concordant with mutations detected in the archival tumor DNA, as all patients with EGFR and KRAS mutations in archival tumor tissue also showed mutations based on analysis of ctDNA when both samples were available (Supplementary Table S2). There was only a single example where a mutation detected in tumor was not detectable in plasma and this was a PIK3CA mutation. In 2 cases KRAS mutations were detected in ctDNA where archival tissue was unavailable for mutation testing. Additional mutations were also detected on-treatment in ctDNA that were not detected at baseline. Specifically, mutational analysis of ctDNA samples from later on-study time points identified a BRAF V600E mutation and, in a separate sample, a PIK3CA H1047R mutation, that were not detected at baseline in plasma or in archival tumor tissue. These mutations could either represent false positives in the assay, or mutations that arose in response to treatment. It is difficult to distinguish between these possibilities as a fresh biopsy was

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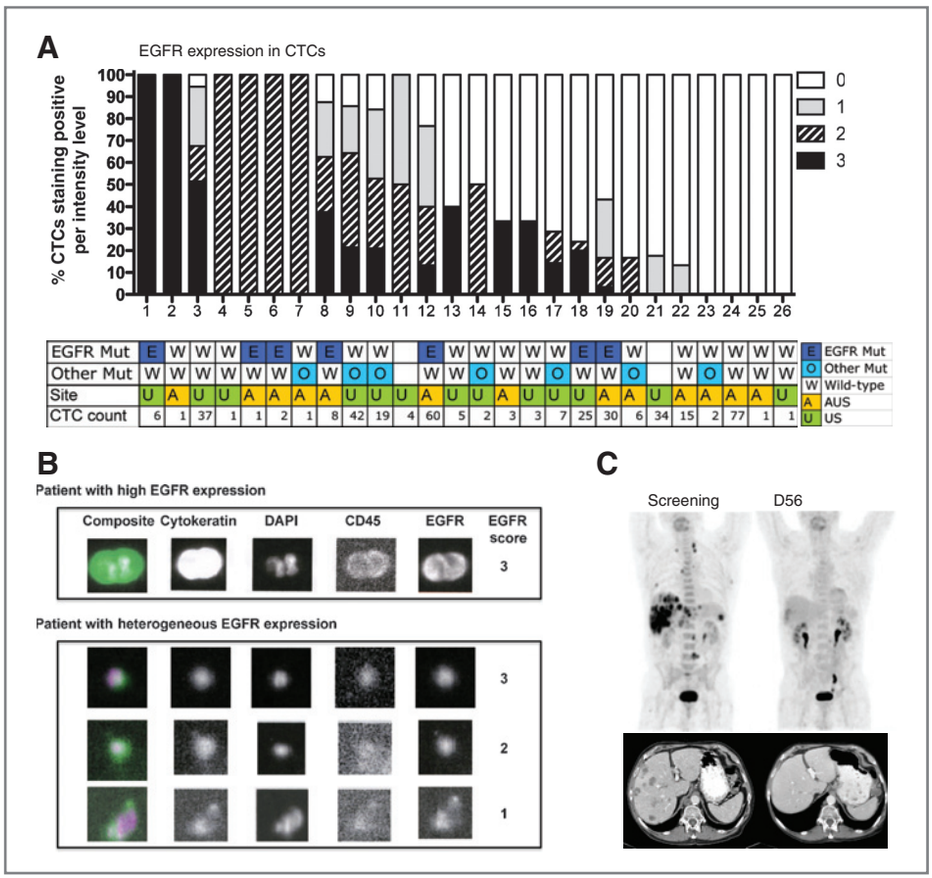

Figure 2. Molecular characterization of CTCs. A, EGFR expression in CTCs listed by patient and graphed as immunofluorescence (IF) staining intensity (0–3, low to high). EGFR Mut, EGFR mutation; Other Mut, mutations in KRAS, BRAF, or PIK3CA; W, wild-type for the mutation; AUS and US are samples shipped from Australia or United States, respectively. B, representative images of a patient with high EGFR expression by IF in CTCs (top) and a patient with heterogeneous EGFR expression in CTCs. C, FDG-PET (top) and CT scans (bottom) showing a partial response in a patient with EGFR mutations detected in tumor DNA, CTCs, and ctDNA. DAPI, 4′,6-diamidino-2-phenylindole.

not available in these instances to independently confirm the presence of the mutations in tumor tissue. The full list of mutations detected either in tumor DNA or plasma or CTCs are shown in Supplementary Table S2.

Correlating treatment effects and genotype with CTC counts and ctDNA levels

CTC numbers were quantitated as described in the methods. Overall, a statistically significant decrease was seen at all later time points compared with baseline (Fig. 3A), suggesting that the combination of pertuzumab and erlotinib treatment may have reduced CTC burden in these patients. This effect was only statistically significant at the D56 time point ($P = 0.058$, D0 vs. D14; $P = 0.056$, D0 vs. D28; $P = 0.018$, D0 vs. D56 by Mann–Whitney U test). A similar treatment effect was observed when using threshold cutoff values of patients with CTC ≥ 1, CTC ≥ 5, or CTC ≥ 10 (Supplementary Table S3). The treatment effects observed on CTC counts were independent of country of origin (Australia or United States) or other patient characteristics. When baseline CTC counts were stratified by mutation status (Fig. 3B), a trend toward higher CTC counts was observed in patients with detectable oncogenic mutations in either EGFR ($P = 0.062$) or KRAS ($P = 0.065$) relative to patients without mutations in these genes in either archival tissue or ctDNA. Patients with EGFR mutations showed the most substantial decreases in CTC counts over the course of treatment ($P = 0.021$, Mann–Whitney U test) and 3 of 7 patients showed decreases of 20 CTCs or more (Fig. 3C). In contrast, on-treatment decreases in CTC counts of similar magnitude were not observed in the KRAS mutant group and only in one of 16 patients wild-type for these mutations. Treatment effects on mutational load in ctDNA were also observed and appeared to be associated with EGFR status but not KRAS status, as all 4 patients with archival tumor EGFR mutations showed evidence of decreased copy number of EGFR mutations in ctDNA based on ΔC_t comparisons, whereas changes of this magnitude were not observed in DNA from patients with KRAS mutations (Fig. 3D). All 4 patients with EGFR mutations who showed decreased mutational load upon treatment had partial responses, whereas the 3 KRAS mutant patients were all nonresponders.

Correlation between CTC counts and radiographic response

CTC counts for correlation with FDG-PET imaging were available from 23 patients at days 14 or 28 and with CT scans from 17 patients at the day 56 time point. Several studies, including a recent report in lung cancer, have indicated that presence of 5 or more CTCs at baseline is indicative of poor prognosis in patients receiving standard

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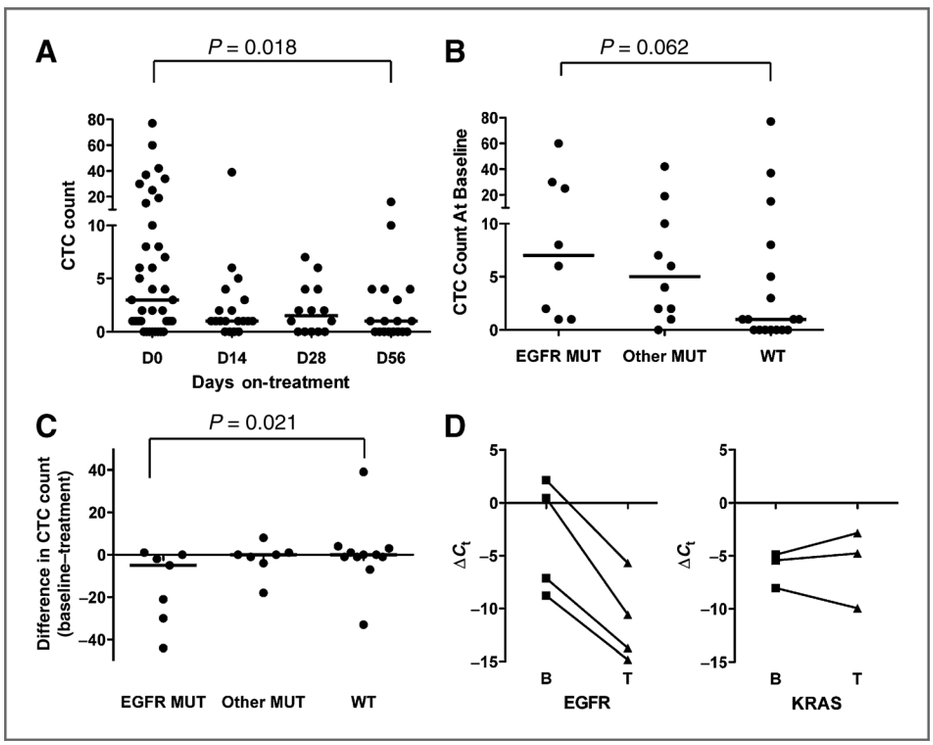

Figure 3. A, CTC counts at baseline and over the course of therapy. B, CTC counts at baseline grouped by genotype. Other MUT indicates mutations in KRAS, BRAF, or PIK3CA. C, changes in CTC count upon treatment grouped by genotype calculated as the difference from last available time point on treatment compared with baseline. D, PCR amplification signal intensity of either EGFR mutation or KRAS mutation plotted in individual patients from sample taken at baseline (B) versus sample taken on treatment (T). Values are plotted as delta cycle threshold or ΔC_t. ΔC_t is computed as CT of EGFR control assay minus CT of EGFR mutant assay. Decreases in ΔC_t indicate decreases in signal from the mutant assay.

chemotherapy regimens (26). On the basis of this, we asked if baseline levels using a cutoff value of $\geq$5 versus <5 CTCs was predictive of either FDG-PET or CT response to the combination of erlotinib and pertuzumab. Using this cutoff value, we detected no association between baseline CTC counts and FDG-PET response at D14/D28 (Fig. 4A). However, patients with 5 or more CTCs at baseline were significantly more likely to have a CT response than patients with less than 5 CTCs at baseline ($P = 0.02$, Mann–Whitney U test; Fig. 4B).

We next grouped patients into responder or nonresponder categories for either FDG-PET response or CT response and looked for differences in baseline CTC levels or on-study changes in CTC levels between groups. We found that patients who showed partial or complete responses (PR or CR) by D14/28 FDG-PET assessments showed a trend toward higher baseline CTC counts (range, 0–37; median, 6) than patients with stable disease or progressive disease (range, 0–19; median, 1; $P = 0.071$, Mann–Whitney U test; Fig. 4C). Despite the high baseline CTC counts, the patients who responded had substantially lower CTC counts at the D14/28 assessment (range, 0–2; median, 0). The decrease in CTC levels in patients who had FDG-PET responses was statistically significant when compared with baseline levels ($P = 0.014$, Mann–Whitney U test, Fig. 4C). Considering CTC changes within individual patients, we found that 5 of 8 patients in the responder group had a 4 or more CTC decrease upon treatment at the D14/D28 time point. In contrast, only 2 of 15 nonresponder patients showed a decrease of this magnitude (Fig. 4D). In addition, 3 patients in the nonresponder group showed increases of 4 or more CTCs at this time point, whereas none of the responder group showed CTC increases.

Our findings were similar when we compared changes in CTC counts with CT response by RECIST. Patients who showed a partial response by CT had higher baseline CTC counts than patients who showed stable disease or progressive disease (Fig. 4E). Despite these higher baseline levels, all 6 patients with a partial response by CT showed at least a 4 CTC decrease at the D56 time point (Fig. 4F). The decrease in CTC counts from baseline in patients with partial response was statistically significant ($P = 0.019$, Mann–Whitney U test). In contrast, only 2 of 11 nonresponder patients showed CTC decreases of this magnitude and the decrease in CTC counts was not significant ($P = 0.239$, Mann–Whitney U test). One of these 2, the patient whose CTC counts decreased from 19 to 1 and had the most significant drop in CTCs in the nonresponder group, had a partial response per RECIST criteria but had other clinical signs of progression and hence was considered a nonresponder per protocol (data not shown).

Association between circulating biomarkers and PFS

Circulating biomarkers evaluated in this study were correlated with the clinical endpoint of PFS using Kaplan–Meier survival analysis. Association of FDG-PET results with survival endpoints has been described previously (27) and will be presented in detail in a report describing the overall clinical study. EGFR mutation status in ctDNA was associated with longer PFS (Fig. 5A). KRAS mutation status in ctDNA was not associated with PFS (Supplementary Fig. S1). Unlike previous studies in patients receiving standard

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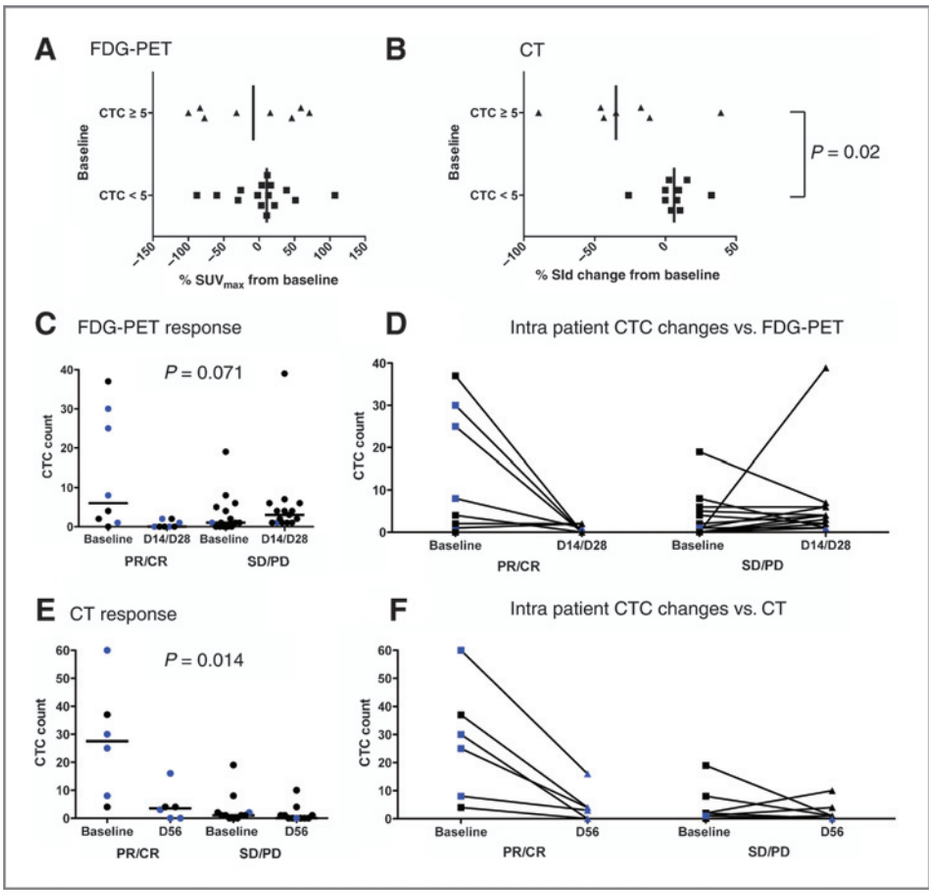

Figure 4. A, baseline CTC counts in patients binned by prognostic cutoff values (favorable as <5 CTCs or unfavorable as ≥5 CTCs) and relationship to response measured either by (A) FDG-PET or (B) CT scans plotted as percentage of change from baseline. SUV_{max}, maximum standard uptake value; SId, sum of the longest unidimensional diameter. CTC counts in responders and nonresponders determined by either FDG-PET in C and D or by CT scans in E or F. D and F, plots of CTC changes in individual patients from baseline to the treatment time point indicated. Responders are defined as patients with partial or complete response (PR/CR) and nonresponders are patients with stable or progressive disease (SD/PD), as described in the text. Blue symbols indicate patients with EGFR mutations.

of care chemotherapy (26), baseline CTC counts using the prognostic cutoff value of less than 5 CTCs in comparison to patients with 5 or more CTC was not predictive of PFS (Supplementary Fig. S2). A likely explanation for this observation is the association of high baseline CTC counts with EGFR mutation status. Decreases in CTC counts upon treatment were associated with longer PFS, showing a trend at early time points and reaching statistical significance at the D56 time point (Fig. 5B and C). We further tested if a composite biomarker that combined changes in CTC counts with CT response would provide further discriminatory value in predicting PFS. Notably, all patients with a CT response by RECIST also had a decrease in CTC counts, and this subset of patients had the longest PFS. Amongst patients who did not have a CT response, decrease in CTC counts was associated with longer PFS than in patients whose CTC counts were unchanged or increased (Fig. 5D).

Discussion

This study evaluated the feasibility of CTC enumeration and molecular analysis in an international, multicenter NSCLC phase II clinical trial. Despite the logistical challenges of conducting a real-time assay in such a setting, meaningful data could be generated: CTCs were detected in the majority of patients (78%), with at least 5 CTCs detected in nearly half the patients (42%). The unique features of this study include the evaluation of CTC changes in the context of a phase II study of targeted agents, as well as parallel examination of CTC and ctDNA levels and dynamic changes in relation to the mutation status of selected oncogenes like EGFR, KRAS, BRAF, and PIK3CA. Our findings indicate that patients with these mutations have a trend toward higher baseline CTC counts than patients who do not have mutations. Importantly, treatment with the erlotinib/pertuzumab combination resulted in a conversion from high baseline CTC counts to low levels in a subset of patients, in particular patients with EGFR mutant tumors. On-treatment decreases in CTC counts were strongly correlated with radiographic response using 2 different imaging modalities, as decreases in CTC counts of 4 or more CTC were associated with both CT response by RECIST criteria as well as metabolic response as determined by FDG-PET. CTC decreases at D56 relative to baseline were associated with longer PFS in this study, and earlier decreases in CTCs at days 14 and 28 showed a trend towards predicting PFS. When CTC changes were used as a composite with CT response, patients who were nonresponders by CT at D56 but had a decrease in CTCs had improved PFS, suggesting potential value in using CTC changes in addition to CT response to predict PFS. However, it should be noted that the number of patients with samples at both time points

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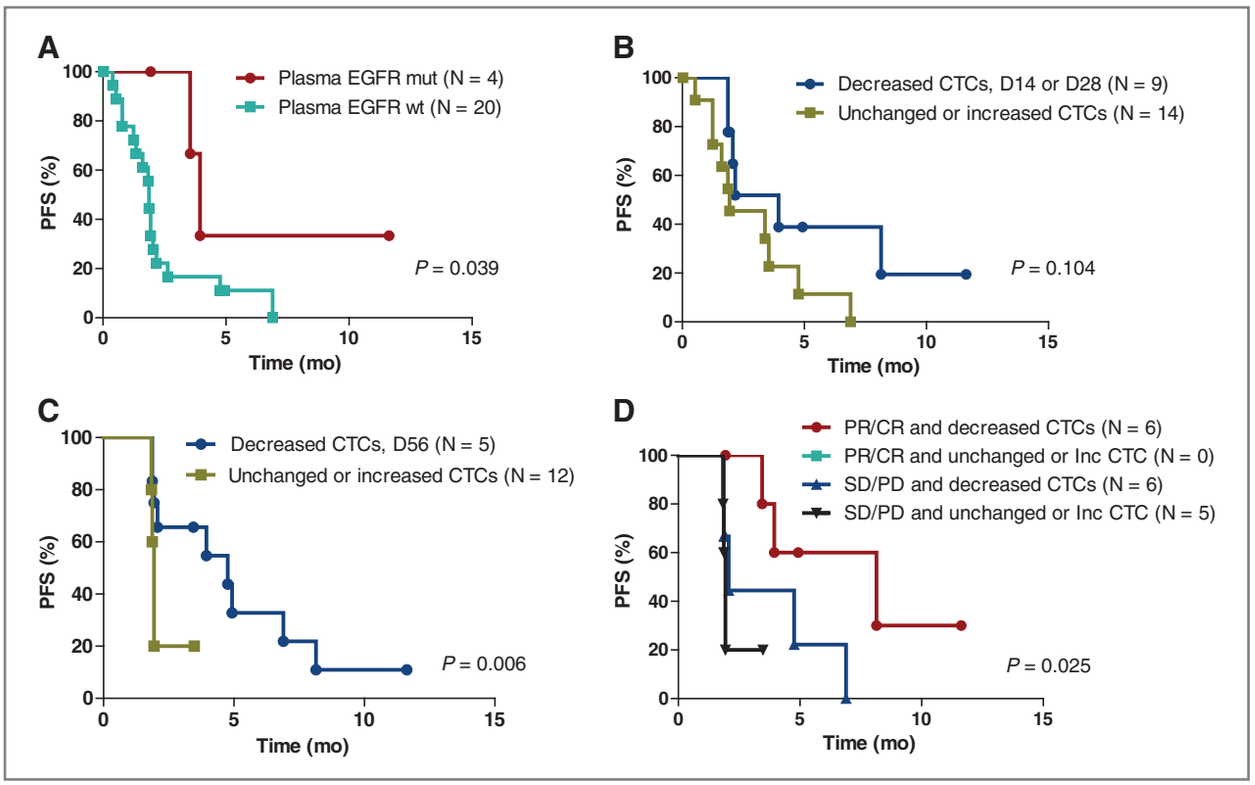

Figure 5. EGFR mutation status in ctDNA and decreases in CTC counts upon treatment are associated with longer PFS. Kaplan–Meier analysis of PFS in patients who had (A) EGFR mutations in ctDNA in comparison with wild-type (B) changes in CTC levels at the D14/D28 early time points from baseline (C) D56 time point and (D) a composite of changes in CTCs levels and CT response at D56. EGFR MUT is EGFR mutant and EGFR WT is wild-type for EGFR; PR/CR, partial or complete response, SD/PD, stable disease or progressive disease by CT scan. Gehan–Breslow–Wilcoxon test was used to determine *P* values in A–C and the log-rank test for trend was used in D.

was small and the conclusions must be tempered by the retrospective nature of the analysis. Because of the single-arm nature of the study, it is unclear whether these findings are due to the administration of erlotinib, an approved agent with known activity in patients with EGFR mutations, or the combination of erlotinib and pertuzumab.

We also evaluated the feasibility of phenotypic analysis in captured CTCs by evaluating EGFR expression. Although EGFR expression levels were not correlated with clinical benefit, the CTC-based target expression results show proof of concept that cell surface expression of receptor tyrosine kinases in CTCs is feasible in NSCLC. Such analyses may aid predictive biomarker studies in cases where expression levels of a surface molecule may be predictive of therapeutic activity.

In addition, we conducted a side-by-side comparison of mutation analysis from DNA extracted from CTCs versus plasma. Our findings indicated that mutational analysis of CTCs captured on the CellSearch platform is challenging, as only one out of 8 EGFR mutations identified in archival tissue and with matched blood sample available for CTC evaluation was detected in CTCs. A possible explanation for this is that the mutation assays used in this study have a sensitivity of 1% to 5% in a background of wild-type DNA (20), so wild-type copies of the gene of interest from contaminating white blood cells coisolated with CTCs using the CellSearch system might obscure the signal from CTCs. We have previously shown that the typical leukocyte range after capture is 1,000 to 3,000 cells (20), which might have hampered detection of CTC-specific mutations. In contrast, mutational analyses from ctDNA showed greater sensitivity in identifying EFGR mutations predicted from archival tumor analyses, as in all evaluable cases the expected mutation was detected. We found that decreases in EGFR mutational load in ctDNA were associated with tumor responses by CT and FDG-PET, suggesting potential utility for such assays in monitoring response to therapy. In addition, plasma analysis identified additional oncogenic mutations in patients with insufficient or unevaluable tissue for mutational analysis, suggesting possible utility of ctDNA for diagnostic screening applications. Intriguingly, serial analyses identified additional mutations in BRAF and PIK3CA that were not detected at baseline, suggesting potential applicability in determining mutation status when patient tumor material is unavailable, or was obtained before therapeutic interventions that could alter tumor genotype and lead to acquired resistance. Analysis of serial biopsies has suggested that multiple resistance mechanism, including but not limited to secondary

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T790M mutations in EGFR or MET amplification, can occur in patients who develop clinical resistance to erlotinib in patients with NSCLC with EGFR mutations (13). As it is generally not practical to collect serial biopsies, longitudinal analysis of resistance mechanisms from CTCs and/or ctDNA could have a role in pinpointing specific resistance mechanisms and selecting a relevant follow-on therapy. Our results suggest that both CTCs and ctDNA analyses could play a role in this effort, as CTCs are amenable to cell-based assays, whereas ctDNA seems to be superior for detecting mutations in circulation. A caveat is that our conclusions on CTCs in this study are limited to the CellSearch platform, and other CTC platforms such as the CTC biochip have reported great sensitivity and specificity for mutational analyses (24).

The prognostic significance of CTC counts is now well documented in multiple indications by the CellSearch assay, where CTC counts above a threshold ($\geq$5 or $\geq$3 CTCs) define an unfavorable prognostic group. Prospective studies led to its U.S. Food and Drug Administration (FDA) clearance as a prognostic assay in breast, colorectal, and prostate cancer (14, 15, 17–19). While these studies showed the clinical validity of the CellSearch assay as a prognostic tool in patients receiving standard chemotherapy regimens, additional clinical applications have yet to be clinically validated. One such application would be the use of changes in CTC levels as a surrogate endpoint that could provide an early indication of antitumor activity. Having such a surrogate endpoint could aid clinical decision making and drug development timelines in early-phase trials where it is challenging to assess OS due to lengthy timelines. Longer term, with appropriate prospective validation in randomized clinical trials to show association with OS, changes in CTC counts could potentially be used in patient management to allow early identification of ineffective therapy and switching to more appropriate therapies. In this study, we provide the first evidence in patients treated with a targeted therapy combination that decreases in CTC counts are correlated with FDG-PET and CT responses, as well as PFS, and thus may be predictive of response to therapy in patients with advanced NSCLC. It should be noted that greatest activity of the pertuzumab/erlotinib combination, with concomitant CTC changes, was seen in patients with EGFR mutations, a known predictor of benefit to anti-EGFR–targeted therapy, and for that reason further study is required to show applicability in the setting of chemotherapy or other targeted therapies in NSCLC. Another notable aspect of the study that differs from previous CTC studies is that high baseline CTC counts were associated with response by RECIST, whereas high CTC counts are normally a poor prognostic factor (26). This observation may be explained by the fact that higher CTC counts in this study were associated with a predictive biomarker, EGFR mutation status.

Our study highlights some of the challenges around successfully incorporating biomarker analyses and novel technologies in early-phase clinical trials. A notable challenge to the use of CTCs for biomarker evaluations is that many patients, even with late-stage cancers, do not have any detectable CTCs using the widely available CellSearch platform. Novel technologies have reported a higher prevalence of CTCs, but commercially available versions of these platforms do not outperform CellSearch in our experience (20). A key limitation of the CellSearch assay is that enrichment of EpCAM-positive CTCs results in the loss of CTCs that have downregulated EpCAM because of epithelial–mesenchymal transition (EMT). Further improvements in CTC capture methods to also include tumor cells that have undergone EMT will likely improve CTC counts and the sensitivity of CTC enumeration as a surrogate marker of clinical activity. A second major challenge to the successful implementation of biomarker studies is that biomarker analyses must be prespecified and included as coprimary or secondary endpoints in a clinical study in order for robust conclusions to be drawn. Even in such cases, subsequent prospective validation of findings in a confirmatory study is required. In this study, CTC biomarker analyses were an exploratory endpoint. Patients who left the study due to clinical progression or safety reasons were not monitored for CTC levels due to the exploratory nature of the analyses, perhaps introducing bias and certainly limiting the number of evaluable patients. Nonetheless, such efforts can be hypothesis generating, and our findings suggest that further study into the role of CTC changes and ctDNA characterization is warranted and will hopefully inform the design of future prospectively designed studies. Successful validation in future studies would be enhanced by designing trials of larger size, inclusion of a control arm, and ensuring samples for biomarker analyses are collected from all patients at all time points. Though our results are from a small phase II clinical trial and await further confirmation, they offer the possibility that CTC enumeration can serve as surrogate marker of clinical activity for targeted therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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