

## Noninvasive Detection of Response and Resistance in *EGFR*-Mutant Lung Cancer Using Quantitative Next-Generation Genotyping of Cell-Free Plasma DNA

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### Abstract

**Purpose:** Tumor genotyping using cell-free plasma DNA (cfDNA) has the potential to allow noninvasive assessment of tumor biology, yet many existing assays are cumbersome and vulnerable to false-positive results. We sought to determine whether droplet digital PCR (ddPCR) of cfDNA would allow highly specific and quantitative assessment of tumor genotype.

**Experimental Design:** ddPCR assays for *EGFR*, *KRAS*, and *BRAF* mutations were developed using plasma collected from patients with advanced lung cancer or melanoma of a known tumor genotype. Sensitivity and specificity were determined using cancers with nonoverlapping genotypes as positive and negative controls. Serial assessment of response and resistance was studied in patients with *EGFR*-mutant lung cancer on a prospective trial of erlotinib.

**Results:** We identified a reference range for *EGFR* L858R and exon 19 deletions in specimens from *KRAS*-mutant lung cancer, allowing identification of candidate thresholds with high sensitivity and 100% specificity. Received operative characteristic curve analysis of four assays demonstrated an area under the curve in the range of 0.80 to 0.94. Sensitivity improved in specimens with optimal cfDNA concentrations. Serial plasma genotyping of *EGFR*-mutant lung cancer on erlotinib demonstrated pretreatment detection of *EGFR* mutations, complete plasma response in most cases, and increasing levels of *EGFR* T790M emerging before objective progression.

**Conclusions:** Noninvasive genotyping of cfDNA using ddPCR demonstrates assay qualities that could allow effective translation into a clinical diagnostic. Serial quantification of plasma genotype allows noninvasive assessment of response and resistance, including detection of resistance mutations up to 16 weeks before radiographic progression. *Clin Cancer Res*; 20(6); 1698–705. ©2014 AACR.

### Introduction

Tumor genotyping has proven to be an invaluable biomarker for identifying subsets of solid tumors with unique sensitivity to targeted therapies. Non-small cell lung cancer (NSCLC) harboring *EGFR* and *ALK* mutations and melanomas harboring *BRAF* mutations have been shown to be highly sensitive to targeted kinase inhibition (1–3). *KRAS* mutations have similarly been shown to have a negative predictive value in identifying cancers that will not respond

to *EGFR* antibodies and *EGFR* kinase inhibitors (4, 5). With innumerable new genotypic biomarkers in development, the power of cancer genomics may become limited only by the availability of biopsy specimens for genotyping. Furthermore, the challenges of genotype-directed cancer care grow even greater when rebiopsy is needed to characterize and target specific resistance mechanisms.

Noninvasive techniques for tumor genotyping may be needed to fully realize the potential of genotype-directed cancer care. Early research suggested that circulating tumor cell capture and analysis had potential as a noninvasive marker of tumor genotype (6); however, clinical development of these technologies has been slow. Several studies have now suggested that highly sensitive genotyping assays can detect mutations in cell-free plasma DNA (cfDNA) from patients with cancer, potentially reflecting the biology of a patient's cancer (7–10). Unfortunately, a challenge of highly sensitive genotyping assays is the detection of low-prevalence mutant alleles of uncertain clinical significance. In a recent study, lung cancers positive for *EGFR* mutations only with a highly sensitive tumor genotyping assay did not

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### Translational Relevance

A major limitation in the advancement of genotype-directed therapy in solid tumors is the challenge of tumor rebiopsy to characterize resistance to targeted therapies. This challenge is particularly important for *EGFR*-mutant lung cancer, in which the T790M resistance mutation is a target of active pharmaceutical development. Here, we demonstrate detection and monitoring of *EGFR* sensitizing and drug resistance mutations in cell-free plasma DNA from patients with *EGFR*-mutant lung cancer. In patients receiving first-line erlotinib, T790M-mediated acquired resistance could be detected up to 16 weeks before radiographic progression; in 1 patient, response of plasma *EGFR* T790M was seen with treatment on a subsequent clinical trial. These data suggest that noninvasive genotyping of cell-free plasma DNA has potential as a clinical biomarker for personalizing therapy of genotype-defined solid tumors.

demonstrate the expected durable benefit from *EGFR* kinase inhibitors, suggesting detection of false positives or mutations present in minor populations (11). The challenge of false-positive results is even greater when studying plasma cfDNA: because cfDNA is mostly of germline origin from ruptured benign cells, tumor-derived mutations are inherently present at a low prevalence, lowering the signal-to-noise ratio of any detection assay.

Toward the goal of identifying an assay for noninvasive genotyping that has a high positive predictive value (PPV), is applicable to multiple genotype-defined solid tumors, and can be easily translated into clinical laboratories, we evaluated cfDNA genotyping using droplet digital PCR (ddPCR). By using a quantitative assay, we aimed to develop a biomarker both for accurate diagnosis of a targetable tumor genotype as well as for convenient monitoring of disease status.

### Materials and Methods

#### Patient population

For our primary study population, we selected patients with advanced NSCLC undergoing routine tumor genotyp-

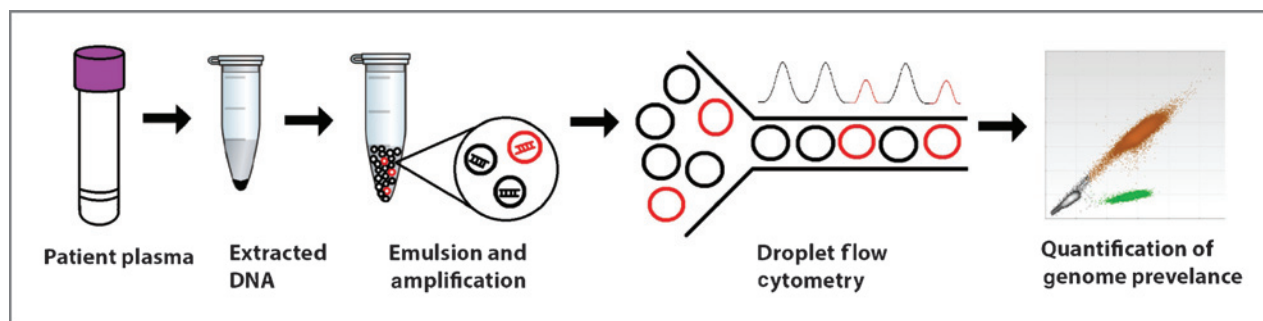
ing in our clinic. All patients consented to an Institutional Review Board (IRB)-approved protocol allowing collection and genomic analysis of blood specimens, limited to <50 mL of blood over any 3-month period. Patients were eligible for cfDNA analysis if they harbored a known *EGFR* or *KRAS* mutation in their NSCLC. Tumor genotyping of *EGFR* and *KRAS* was performed in a clinical, Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. A second population of patients with advanced melanoma and a known *BRAF* genotype was also studied after consent to specimen collection on an IRB-approved protocol.

#### Plasma collection

For each eligible patient, plasma was collected during routine care either before first-line therapy or at a subsequent time when the cancer was progressing on therapy. Additional follow-up specimens were collected if possible during routine care. Each specimen was collected into one 10-mL EDTA-containing vacutainer and was spun into plasma within 4 hours of collection. cfDNA was extracted from 2 mL of plasma, and the final DNA eluent (approximately 100  $\mu$ L) was frozen at  $-80^{\circ}\text{C}$  until genotyping (Supplementary Materials and Methods). Mean isolated DNA mass per 1 mL of plasma across all samples was 91.5 ng of DNA (interquartile range, 57–305 ng), quantified by PicoGreen as per the manufacturer's recommendation.

#### Droplet Digital PCR

ddPCR is a digital PCR technology that takes advantage of recent developments in microfluidics and surfactant chemistries. Whereas conventional digital PCR involves a sometimes cumbersome process of diluting input DNA into individual wells for analysis (12, 13), ddPCR emulsifies input DNA into thousands of droplets that are PCR amplified and fluorescently labeled, and then read in an automated droplet flow cytometer (Fig. 1; ref. 14). Each droplet is individually assigned a positive or negative value based on the fluorescent intensity. The number of positive and negative droplets is read by a flow cytometer and is used to calculate the concentration of an allele. To minimize bias and to ensure the integrity of results, the laboratory was blinded to the tumor genotype when testing plasma specimens, but results were selectively unblinded for data



**Figure 1.** Plasma genotyping using ddPCR. cfDNA is extracted from a plasma specimen and emulsified with oil into thousands of droplets, each containing approximately 0 to 1 molecules of target DNA. PCR is performed to endpoint in each droplet. These droplets are run through a flow cytometer, in which droplets containing mutant and wild-type DNA emit different colored signals. The count of these signals allows quantification of allelic prevalence.

analysis. A detailed protocol for each ddPCR assay is provided in the Supplementary Materials and Methods. Each plasma sample was analyzed in triplicate with an increasing quantity of input DNA (e.g., 1, 2, and 4  $\mu$ L) on a QX100 digital droplet reader (Supplementary Materials and Methods). Results were reported as copies of mutant allele per mL of plasma, as done by prior investigators (9, 10).

## Results

### Assay characteristics

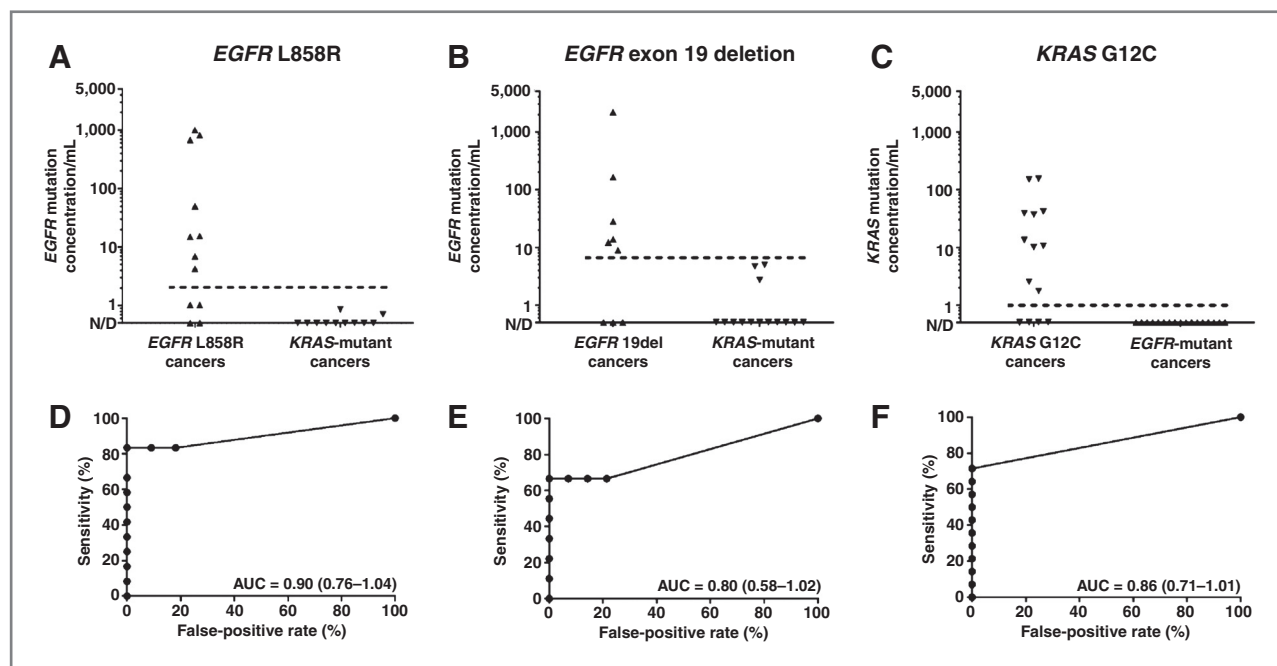
We first developed two assays for *EGFR* L858R and exon 19 deletions; the latter assay was designed to detect loss of the wild-type signal and therefore could detect exon 19 deletions of variable sequence. Specifically, the assay is designed in such manner that a VIC<sup>®</sup>-labeled "reference probe" sequence is shared by both the wild-type and the deletion mutants, whereas the FAM<sup>™</sup>-labeled probe sequence spans the hotspots of the deletion and thus is only present in wild-type samples (13). An *EGFR* exon 19 wild-type sample will therefore show both FAM<sup>™</sup>- and VIC<sup>®</sup>-labeled droplets, whereas an *EGFR* exon 19 mutant will only have VIC<sup>®</sup>-labeled droplets. To demonstrate the analytic sensitivity and specificity of each assay, each ddPCR cycling condition was optimized to yield the maximum fluorescent signal with minimal increase in background signal (Supplementary Fig. S1). Using serial dilutions of mutant DNA, we found that ddPCR detects mutation prevalence between 0.005% and 0.01%, with a sensitivity of 5 to 50 mutant copies in a background of 10,000 wild-type copies (Supplementary Fig. S2), depending on the mutation

assayed. Experiments were repeated over 3 nonconsecutive days. Both assays demonstrated linear quantification of allelic prevalence across a dynamic range spanning 4 orders of magnitude. From a technical standpoint, this suggests that ddPCR provides a reliable and quantitative measure of low-prevalence *EGFR* mutant alleles within a plasma sample.

### Maximizing positive predictive value

To optimize the specificity of our *EGFR* genotyping assays (and utility in guiding clinical decisions), we tested the incidence of false-positive reads in a gold-standard negative population. To ensure selection of patients certain to be wild-type for *EGFR*, we studied patients with *KRAS*-mutant lung cancers. Large studies have found that *EGFR* and *KRAS* mutations are nonoverlapping in NSCLC and represent distinct cancer populations (15, 16). Furthermore, *KRAS*-mutant lung cancers are recognized to be insensitive to treatment with *EGFR* kinase inhibitors (5, 17); although small subpopulations of cells within an individual *KRAS*-mutant lung cancer might hypothetically harbor mutations in *EGFR*, they evidently do not affect drug sensitivity for these cancers. Therefore, any *EGFR*-mutant DNA found in the plasma of patients with *KRAS*-mutant NSCLC can be interpreted as biologically insignificant and representative of the "reference range" for our assay.

We first studied the *EGFR* L858R assay in 23 patients with NSCLC, 12 with *EGFR* L858R, and 11 with *KRAS* mutations in their cancers. Low levels of *EGFR* L858R were detected in 2 *KRAS*-mutant cases (18%) with a peak level of 0.9 copies/mL (Fig. 2A). Using 1 copy/mL as our threshold



**Figure 2.** Detection of mutant alleles in gold-standard positive and negative populations, using assays for *EGFR* L858R (A), *EGFR* exon 19 deletion (B), and *KRAS* G12C (C). ROC curves are also shown (D–F). By studying plasma from patients with lung cancer with nonoverlapping genotypes, a "reference range" for each assay can be identified. Dashed lines represent one candidate threshold for positive with a very high specificity and acceptable sensitivity.

for a positive result, 8 of 12 cases could be correctly identified as positive for *EGFR* L858R. We next studied the variable exon 19 deletion assay in a new cohort of 23 patients with NSCLC, 9 with *EGFR* exon 19 deletions, and 14 with *KRAS* mutations in their cancers. Low levels of *EGFR* exon 19 deletions were detected in 3 *KRAS*-mutant cases (21%) with a peak value of 5 copies/mL (Fig. 2B). Using 6 copies/mL as our threshold for a positive result, 6 of 9 cases could be correctly identified as positive for *EGFR* exon 19 deletion. Finally, we tested the reverse experiment in 31 patients with NSCLC using a *KRAS* G12C assay that we developed as above. Of 17 patients with *EGFR*-mutant lung cancer, none had measurable mutant *KRAS* (Fig. 2C). Using a threshold of 0.5 copies/mL, 10 of 14 *KRAS* G12C cases could be correctly identified as positive. For each assay, a receiver operating characteristic (ROC) curve was generated, with an area under the curve (AUC) in the range of 0.8 to 0.9 (Fig. 2D–F).

To gauge the generalizability of this assay to other genotype-defined malignancies, we developed an assay for *BRAF* V600E in the fashion described above and tested plasma specimens from 13 patients with melanoma. Using a threshold of 0.5 copies/mL for a positive result, 7 of 8 cases could be correctly identified as positive, and the ROC curve had a high AUC (Supplementary Fig. S3), demonstrating potential value of ddPCR genotyping in a disease other than NSCLC.

#### Quality control to improve sensitivity

To better understand the false-negative results, we measured human long interspersed element 1 (LINE-1) to assess the quantity and quality of cfDNA from the 32 *EGFR*- and *KRAS*-mutant lung cancer cases (true positives) studied in the above experiments. LINE-1 is an easily measured, genomically common retrotransposon that has been previously used to estimate total DNA in plasma (Supplementary Fig. S4; ref. 18). Median LINE-1 concentration was 168 ng/mL (interquartile range, 73–620 ng/mL) across the 32 specimens.

Detection of mutant alleles improved with increased levels of LINE-1 (Fig. 3). Sensitivity was 81% in the 16 cases with LINE-1 levels higher than median, and 50% in the 16 cases with LINE-1 levels below median ( $P = 0.07$ ). However, three outlier cases with the highest levels of LINE-1 (greater than approximately 20,000 ng/mL) had no detectable levels of plasma genotype, likely indicating a high quantity of germline DNA obscuring detection of mutant cfDNA. These results suggest that LINE-1 levels may assist in identifying which plasma specimens are vulnerable to falsely negative genotyping result.

#### Developing a disease-monitoring biomarker

To assess the value of cfDNA genotype prevalence as a disease-monitoring biomarker, we quantified the range of variability. Using the techniques described above, we generated a fifth genotyping assay to detect the *EGFR* T790M mutation. We generated human plasma DNA specimens that contained either 1, 2, 10, or 20 copies of *EGFR* T790M

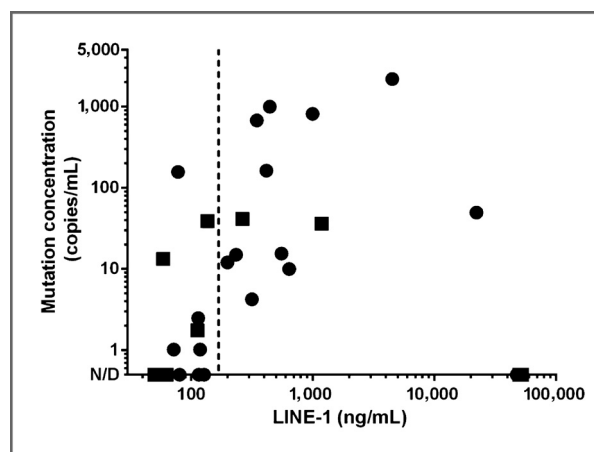


Figure 3. Plasma DNA quantification to optimize sensitivity. Studying genotype concentration in gold-standard positive cases, the false-negative results all have either low or very high levels of LINE-1. Sensitivity is 81% above the median LINE-1 concentration of 168 ng/mL. Circles, *EGFR*-mutant cases; squares, *KRAS*-mutant cases.

per 25- $\mu$ L reaction, divided each into 32 individual specimens, and tested each of these for T790M prevalence by ddPCR. The assay exhibited a Poisson distribution between positives droplets and sample input with acceptable coefficient of variance in the range of 20% to 30% (Supplementary Fig. S5), suggesting that changes exceeding this amount could represent a true change in tumor burden or biology.

To gauge feasibility, we studied serial plasma specimens from patients with genotype-defined lung cancer or melanoma to determine whether changes in cfDNA were representative of tumor biology (Fig. 4). In a patient with *EGFR*-mutant NSCLC receiving chemotherapy after failing erlotinib (Fig. 4A), an increase in plasma L858R and T790M was seen with development of new brain metastases, followed by decreased plasma levels when treatment on a clinical trial was initiated. In a second case of *EGFR*-mutant NSCLC receiving chemotherapy (Fig. 4B), plasma L858R decreased as the patient's pleural drainage resolved, though computed tomography imaging of the nonmeasurable disease showed disease stability. In a patient with *KRAS*-mutant NSCLC and bone metastases (Fig. 4C), chemotherapy caused a decrease in plasma G12C levels concordant with improved pain control and decreased opiate requirement. Finally, a patient with *BRAF*-mutant melanoma had progression on experimental immune therapy followed by response to vemurafenib (Fig. 4D), seen in the increase and decrease of plasma V600E levels. This pilot experience suggests cfDNA genotyping has value for serial assessment of disease status, even in patients without objectively measurable disease on CT.

#### Monitoring for resistance mutations

To determine whether ddPCR could identify the development of resistance mutations after treatment with targeted therapy, we studied patients with advanced *EGFR*-mutant NSCLC treated on a prospective clinical trial of first-

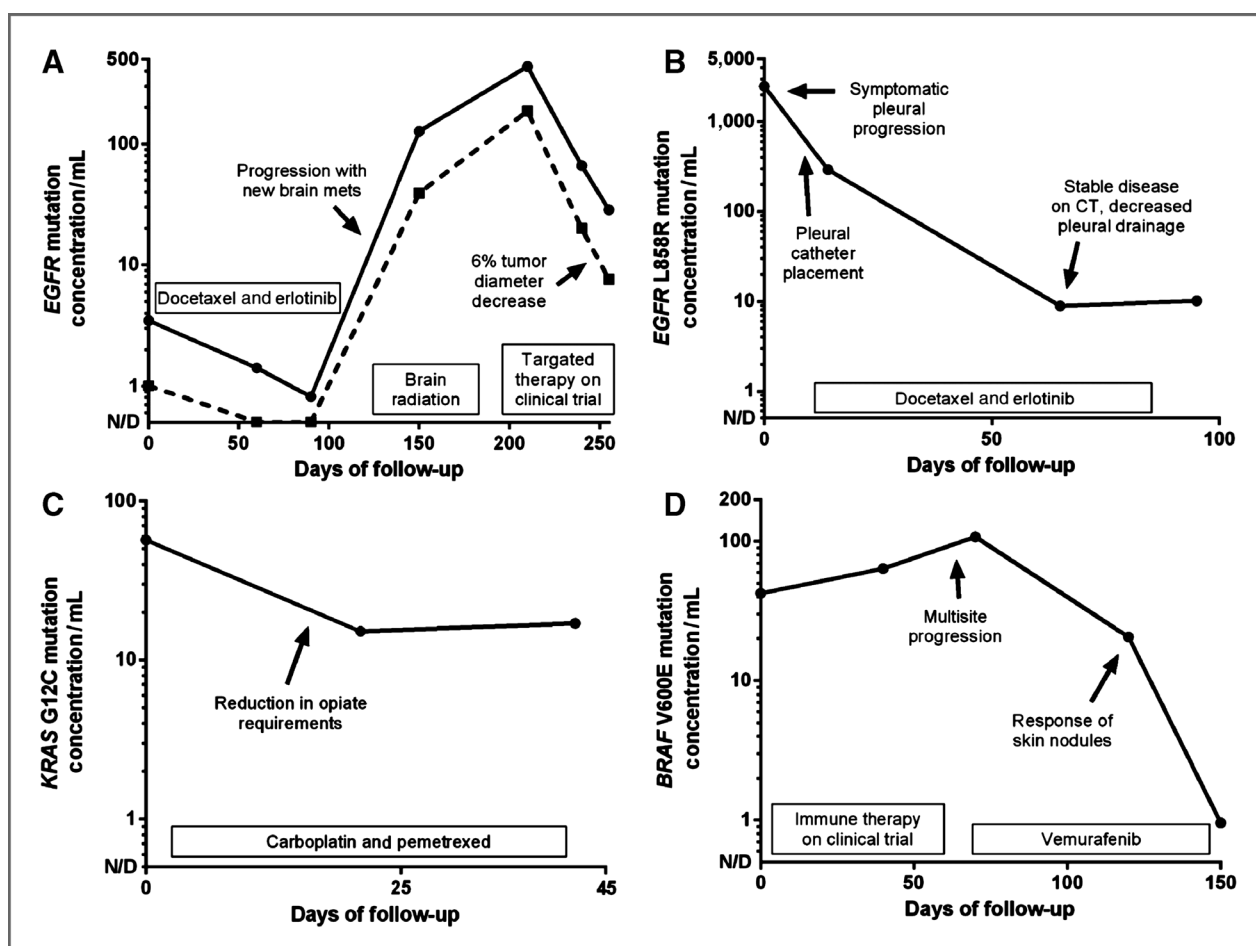


Figure 4. Serial measurement of plasma genotype for disease monitoring. A wide dynamic range is seen in some cases (A and B). Decreases in plasma genotype can be seen both in cases of objective tumor shrinkage (A and D) and in cases of symptomatic response with no measurable disease (B and C). Concurrent *EGFR* L858R (A, solid line) and T790M (A, dashed line) mutations trend in parallel.

line erlotinib (NCT00997334), limiting our analysis to 13 patients that had serial plasma specimens collected until development of objective progression per the Response Evaluation Criteria in Solid Tumors (RECIST). In each of these patients, genotyping of archived tissue at diagnosis identified an *EGFR* exon 19 deletion without evidence of T790M. Four patients had no detectable pretreatment plasma genotype and were excluded, leaving 9 cases (69%) for analysis.

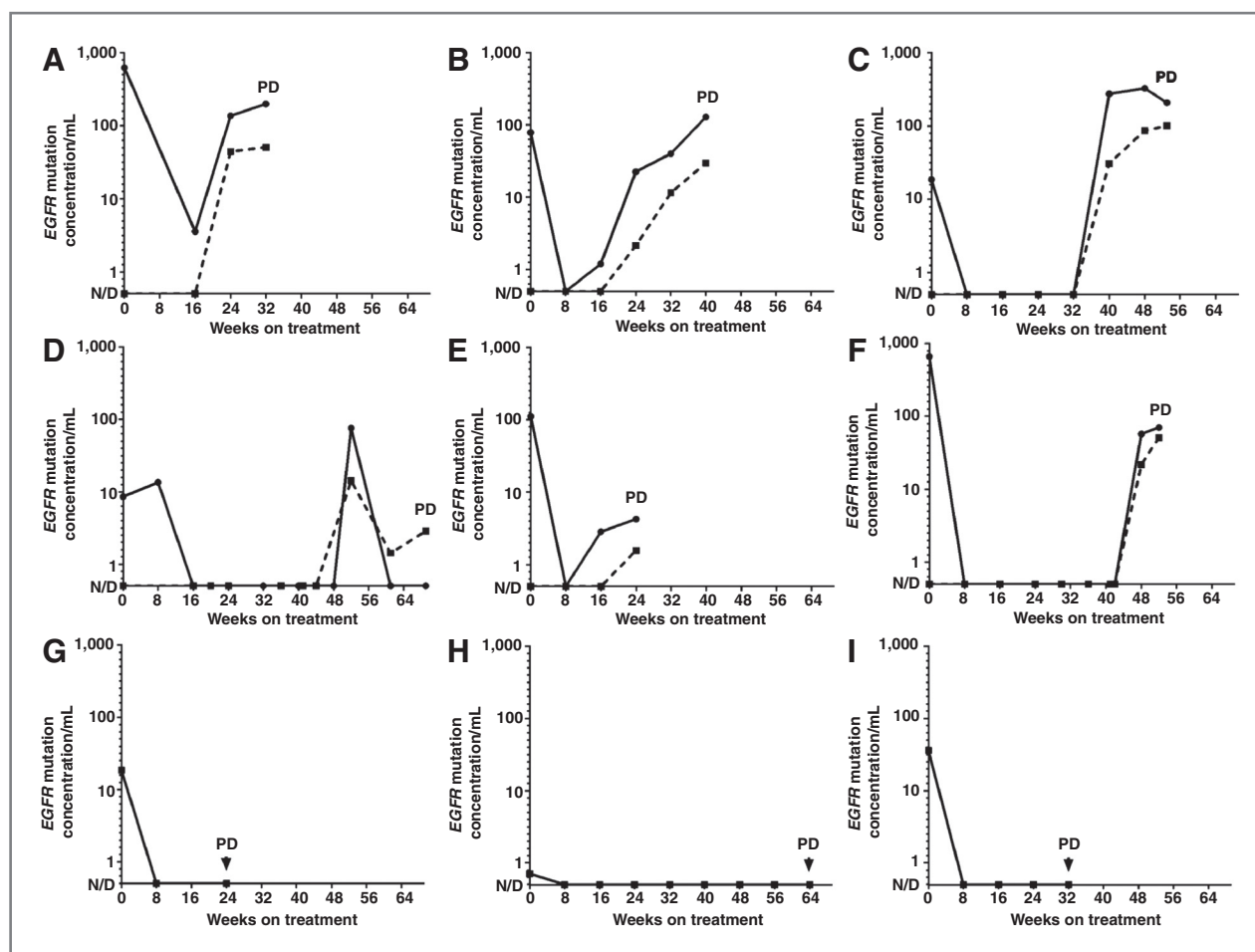
All 9 patients exhibited a plasma response to erlotinib, with 8 demonstrating a complete plasma response (Fig. 5). In 6 of the patients, plasma levels of mutant *EGFR* were again detected at objective progression, with plasma progression detected 4 to 24 weeks before RECIST progression. In each of these patients, plasma T790M could also be identified at progression, generally at somewhat lower levels than the *EGFR* sensitizing mutation. Four of these patients had a tumor rebiopsy adequate for *EGFR* genotyping, and T790M was confirmed in each. The remaining 3 patients had no reemergence of plasma genotype at objective progression; notably, each of these patients had indolent

asymptomatic progression in the chest only, such that they subsequently continued single-agent erlotinib off-protocol.

## Discussion

We herein describe a new quantitative assay for plasma-based tumor genotyping, which has been technically optimized for translation into clinical practice. By quantifying the prevalence of targetable genotypes in clinical plasma specimens, and through study of rigorous gold-standard negative cases harboring nonoverlapping cancer genotypes, we have identified a reference range for *EGFR* and *KRAS* mutation detection using ddPCR. Using such a calculated threshold as the criteria for a positive results, as well as LINE-1 concentration to eliminate poor quality specimens, our data suggest that this assay can have high sensitivity and specificity. These proposed thresholds require prospective validation.

Because many targetable genotypes are relatively uncommon, we have focused our assay development on maximizing specificity. Consider, for example, a plasma assay for detecting *EGFR* sensitizing mutations, present in 8.6% of 10,000 patients with NSCLC from the large French



**Figure 5.** Plasma levels of mutant *EGFR* in 9 patients receiving first-line erlotinib until objective disease progression (PD) by RECIST. In all patients (A–I), plasma levels of the *EGFR* sensitizing mutation (solid line) drop in response to treatment, with 8 patients (B–I) having a complete plasma response. In 6 patients, plasma genotype levels reemerge up to 4 months before disease progression, and a lower concentration of T790M (dashed line) is also detected. In 3 patients (G–I), plasma genotype was not detected at the time of disease progression; all 3 had indolent progression in the chest only.

experience (19). In this population, a plasma assay for *EGFR* mutations with 80% sensitivity and 90% or 95% specificity would have a PPV of only 43% or 60%, respectively. For this reason, a clinical-grade assay will likely need to sacrifice sensitivity to optimize specificity. In the same population, an assay with 70% sensitivity and 99% or 100% specificity would result in a PPV of 87% or 100%, respectively. Furthermore, the need to maximize specificity is magnified when testing for rarer genotypes such as *BRAF* V600E in NSCLC, representing only 2% of patients (20). One valuable characteristic of a quantitative assay such as ddPCR is the flexibility to allow an alteration of the criterion for positive if the pretest probability changes (e.g., Asian patients with lung cancer). This is in contrast to an allele-specific PCR assay, such as one which showed high concordance with tumor genotyping in a preliminary analysis of plasma from 241 Asian patients with lung cancer (21); such an assay is more difficult to quantitate and therefore more difficult to adjust to a higher specificity criterion in populations with lower mutation prevalence.

This study is one of several that have investigated plasma genotyping as a way of noninvasively detecting the *EGFR* T790M resistance mutation in patients with lung cancer treated with *EGFR* kinase inhibitors (13, 22–24). Yet this is the first study to demonstrate, across multiple patients, that serial assessment of plasma genotype allows detection of resistance weeks (and sometimes months) before clinical development of resistance. Early detection of resistance has particular importance given the growing role of *EGFR* T790M as a biomarker for patients with *EGFR*-mutant lung cancer and acquired resistance. First, acquired T790M has been associated with indolent growth and a favorable prognosis compared with T790M-negative acquired resistance (25). Second, third-generation *EGFR* kinase inhibitors with T790M-specific activity have recently been shown to have activity in patients with T790M-mediated acquired resistance (26–28). Although pharmaceutical development of T790M-directed targeted therapies could be limited by the challenges of performing a repeat biopsy after resistance develops (29), our data suggest that early emergence of

*EGFR* T790M can be identified noninvasively using ddPCR, and potentially used to guide subsequent treatment.

The quantitative nature of plasma genotyping with ddPCR also offers a mechanism for monitoring the prevalence of tumor clones harboring a specific genotype, potentially giving insight into the pharmacodynamics of a targeted therapy. In liquid malignancies like chronic myelogenous leukemia, rapidity of molecular response to kinase inhibitors has been established as an important biomarker of prognosis, and helps indicate which patients may need early salvage therapy (30). Diehl and colleagues also found that cfDNA levels have the potential to be used in colorectal cancer, much like carcinoembryonic antigen (CEA) levels, to distinguish successful versus unsuccessful surgical resection (31). Similarly, serial assessment of a plasma genotype may prove to be a valuable biomarker for genotype-defined solid tumors treated with targeted therapies, both as a clinical biomarker of favorable outcome and potentially as an early clinical trial endpoint. Indeed, this was even suggested in our small series—the 1 patient not exhibiting a complete plasma response to erlotinib had early progression—and will need to be studied in larger cohorts. In addition, response assessment using plasma genotype quantification could potentially allow trial accrual for those patients with genotype-defined solid tumors that are not objectively measurable using conventional response criteria.

Although there is currently no standard unit for the reporting of plasma genotyping results, we have reported our results using copies per mL of plasma, as reported previously in the literature (9, 10). Other studies have presented plasma genotyping results as the percent of reactions that are mutant (7, 13). However, we worry that this relative concentration may be less precise, particularly at low concentrations—while 2 mutant copies/2,000 wild-type copies and 20 mutant copies/20,000 wild-type copies both can be calculated as 0.1% mutant, they are not equal, and the former is more likely to be a false positive. To facilitate comparisons, we have also provided our data recalculated using this alternate unit (see Supplementary Data). As this is a dynamically changing field, we encourage other investigators to consider that there may also be other more precise strategies for the presentation of plasma genotyping results.

## References

- Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. *EGFR* mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500.
- Kwak EL, Bang Y-J, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693–703.
- Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated *BRAF* in metastatic melanoma. *N Engl J Med* 2010;363:809–19.
- Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. *K-ras* mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008;359:1757–65.
- Jackman DM, Miller VA, Cioffredi LA, Yeap BY, Janne PA, Riely GJ, et al. Impact of epidermal growth factor receptor and *KRAS* mutations on clinical outcomes in previously untreated non-small cell lung cancer patients: results of an online tumor registry of clinical trials. *Clin Cancer Res* 2009;15:5267–73.
- Maheswaran S, Sequist LV, Nagrath S, Ullkus L, Brannigan B, Collura CV, et al. Detection of mutations in *EGFR* in circulating lung-cancer cells. *N Engl J Med* 2008;359:366–77.
- Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of *KRAS* mutations and acquired resistance to anti-*EGFR* therapy in colorectal cancer. *Nature* 2012;486:532–6.

In conclusion, we herein present a proof of concept demonstrating the clinical utility of cfDNA genotyping for detecting and monitoring *EGFR* sensitizing and drug resistance mutations for patients with NSCLC. ddPCR is an attractive technology as its speed, cost, and ease of use are similar to other PCR-based assays, yet the sensitivity and quantitative nature of this assay offers broader clinical application. Prospective validation based upon this initial experience is needed, and is under way.

## Disclosure of Potential Conflicts of Interest

G.R. Oxnard is a consultant/advisory board member for Astellas, Astra-Zeneca, AVEO, Boehringer Ingelheim, Genentech, and Novartis; and has received honoraria from Astra-Zeneca, Boehringer Ingelheim and Chugai. D.M. Jackman is a consultant/advisory board member for Foundation Medicine and Genentech; and has received honoraria from Chugai. P.A. Janne is a consultant/advisory board member for Abbot, Astra-Zeneca, Boehringer Ingelheim, Chugai, Clovis, Genentech, Pfizer, and Sanofi. G.R. Oxnard, C. P. Paweletz, and P.A. Janne are inventors on a pending patent related to findings described in this manuscript. P.A. Janne is a co-inventor on a patent held by the Dana-Farber Cancer Institute for the use of *EGFR* genotyping, and receives a share of post-market licensing revenue distributed by DFCL.

## Authors' Contributions

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** G.R. Oxnard, C.P. Paweletz, Y. Kuang, A. O'Connell, J.J. Luke, P.A. Janne

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8. Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater SA, Powers P, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. *Clin Cancer Res* 2012;18:3462–9.
9. Diaz LA Jr, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 2012;486:537–40.
10. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* 2013;368:1199–209.
11. Zhou Q, Zhang X-C, Chen Z-H, Yin X-L, Yang J-J, Xu C-R, et al. Relative abundance of EGFR mutations predicts benefit from gefitinib treatment for advanced non-small-cell lung cancer. *J Clin Oncol* 2011;29:3316–21.
12. Vogelstein B, Kinzler KW. Digital PCR. *Proc Natl Acad Sci U S A*. 1999;96:9236–41.
13. Yung TKF, Chan KCA, Mok TSK, Tong J, To K-F, Lo YMD. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. *Clin Cancer Res* 2009;15:2076–84.
14. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital pcr system for absolute quantitation of DNA copy number. *Anal Chem* 2011;83:8604–10.
15. Cardarella S, Ortiz TM, Joshi VA, Butaney M, Jackman DM, Kwiatkowski DJ, et al. The introduction of systematic genomic testing for patients with non-small-cell lung cancer. *J Thorac Oncol* 2012;7:1767–74.
16. Johnson ML, Sima CS, Chaft J, Paik PK, Pao W, Kris MG, et al. Association of KRAS and EGFR mutations with survival in patients with advanced lung adenocarcinomas. *Cancer* 2013;119:356–62.
17. Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, et al. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* 2005;2:e17.
18. Rago C, Huso DL, Diehl F, Karim B, Liu G, Papadopoulos N, et al. Serial assessment of human tumor burdens in mice by the analysis of circulating DNA. *Cancer Res* 2007;67:9364–70.
19. Barlesi F, Blons H, Beau-Faller M, Rouquette I, Ouafik Lh, Mosser J, et al. Biomarkers (BM) France: results of routine EGFR, HER2, KRAS, BRAF, PI3KCA mutations detection and EML4-ALK gene fusion assessment on the first 10,000 non-small cell lung cancer (NSCLC) patients (pts). *J Clin Oncol* 31, 2013 (suppl; abstr 8000).
20. Cardarella S, Ogino A, Nishino M, Butaney M, Shen J, Lydon C, et al. Clinical, pathologic, and biologic features associated with BRAF mutations in non-small cell lung cancer. *Clin Cancer Res* 2013;19:4532–40.
21. Mok T, Wu YL, Lee JS, Yu C-J, Sriuranpong V, Wen W, et al. Detection of EGFR-activating mutations from plasma DNA as a potent predictor of survival outcomes in FASTACT 2: A randomized phase III study on intercalated combination of erlotinib (E) and chemotherapy (C). *J Clin Oncol* 31, 2013 (suppl; abstr 8021).
22. Murtaza M, Dawson S-J, Tsui DWY, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* 2013;497:108–12.
23. Nakamura T, Sueoka-Aragane N, Iwanaga K, Sato A, Komiya K, Kobayashi N, et al. Application of a highly sensitive detection system for epidermal growth factor receptor mutations in plasma DNA. *J Thorac Oncol* 2012;7:1369–81.
24. Sakai K, Horiike A, Irwin DL, Kudo K, Fujita Y, Tanimoto A, et al. Detection of epidermal growth factor receptor T790M mutation in plasma DNA from patients refractory to epidermal growth factor receptor tyrosine kinase inhibitor. *Cancer Sci* 2013;104:1198–204.
25. Oxnard GR, Arcila ME, Sima CS, Riely GJ, Chmielecki J, Kris MG, et al. Acquired resistance to EGFR tyrosine kinase inhibitors in EGFR mutant lung cancer: distinct natural history of patients with tumors harboring the T790M mutation. *Clin Cancer Res* 2011;17:1616–22.
26. Zhou W, Ercan D, Chen L, Yun CH, Li D, Capelletti M, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature* 2009;462:1070–4.
27. Sequist LV, Soria J-C, Gadgeel SM, Wakelee HA, Camidge DR, Varga A, et al. First-in-human evaluation of CO-1686, an irreversible, selective, and potent tyrosine kinase inhibitor of EGFR T790M. *J Clin Oncol* 31, 2013 (suppl; abstr 2524).
28. Ranson M, Pao W, Kim D-W, Kim S-W, Ohe Y, Filip E, et al. AZD9291: an irreversible, potent and selective tyrosine kinase inhibitor (TKI) of activating (EGFRm+) and resistance (T790M) mutations in advanced NSCLC. *J Thorac Oncol* 2013;8:MO21.12.
29. Arcila ME, Oxnard GR, Nafa K, Riely GJ, Solomon SB, Zakowski M, et al. Rebiopsy of lung cancer patients with acquired resistance to EGFR inhibitors and enhanced detection of the T790M mutation using a locked nucleic acid-based assay. *Clin Cancer Res* 2011;17:1169–80.
30. Branford S, Kim DW, Soverini S, Haque A, Shou Y, Woodman RC, et al. Initial molecular response at 3 months may predict both response and event-free survival at 24 months in imatinib-resistant or -intolerant patients with Philadelphia chromosome-positive chronic myeloid leukemia in chronic phase treated with nilotinib. *J Clin Oncol* 2012;30:4323–9.
31. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med* 2008;14:985–90.



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