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TITLE:Noninvasive Personalization of Lung Cancer Therapy Using a New, Clinical-Grade Assay for Plasma-Based Measurement and Monitoring of Tumor Genotype

PRINCIPAL INVESTIGATOR: Geoffrey Oxnard, MD

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston MA, 02115-5418

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5d. PROJECT NUMBER

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
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14. ABSTRACT
The overarching aim of this project was to perform blinded prospective validation of a new assay for noninvasive and quantitative genotyping of cell-free plasma DNA (cfDNA) using droplet digital PCR (ddPCR). In this project we launched a prospective correlative study to validate the accuracy of this assay for detection of EGFR and KRAS mutations in patients with NSCLC, to optimize handling conditions to maximize the reliability of results, and to understand response of plasma cfDNA on therapy. We successfully validated the assay with 100% specificity for key EGFR and KRAS driver mutations. Assay sensitivity was 64-83%, and improved with a greater number of sites of metastatic disease. Serial plasma assessment revealed dramatic early reductions in plasma mutation levels, which predicted for a response on imaging. Importantly, one of the most important successes of this project was using the validation of this assay to lay the groundwork for my career development as the awardee, who has received grant funding to pursue multiple follow-up projects using this technology.

15. SUBJECT TERMS
Non-small cell lung cancer, liquid biopsy, plasma genotyping, EGFR, KRAS, targeted therapy

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1. **INTRODUCTION:** The overarching aim of this project was to perform blinded prospective validation of a new assay for noninvasive and quantitative genotyping of cell-free plasma DNA (cfDNA) using droplet digital PCR (ddPCR). In this project we launched a prospective correlative study to validate the accuracy of this assay for detection of EGFR and KRAS mutations in patients with NSCLC, to optimize handling conditions to maximize the reliability of results, and to understand response of plasma cfDNA on therapy. We successfully validated the assay with 100% specificity for key EGFR and KRAS driver mutations. Assay sensitivity was 64-83%, and improved with a greater number of sites of metastatic disease. Serial plasma assessment revealed dramatic early reductions in plasma mutation levels, which predicted for a response on imaging. Importantly, one of the most important successes of this project was using the validation of this assay to lay the groundwork for my career development as the awardee, who has received grant funding to pursue multiple follow-up projects using this technology.

2. **KEYWORDS:**

   Non-small cell lung cancer, liquid biopsy, plasma genotyping, EGFR, KRAS, targeted therapy

3. **ACCOMPLISHMENTS:**

a. **What were the major goals of the project?**

There were 3 aims to this grant stated in the SOW:

i. To prospectively validate the accuracy of plasma genotyping (A) in patients with newly diagnosed NSCLC undergoing tumor genotyping and (B) in patients with acquired resistance to erlotinib undergoing tumor rebiopsy

- Enrollment of 180 patients to this analysis completed on 4/22/2015. This includes 120 patients with newly diagnosed NSCLC (Aim 1A, Cohort 1A on our trial) and 60 patients with acquired resistance to EGFR TKI (Aim 1B, Cohort 2A on our trial). 169 of these patients had paired plasma ddPCR and tumor genotyping available and were eligible for the final accuracy analysis published in JAMA Oncology.

ii. To develop criteria for plasma response that allow prediction of early treatment failure in patients with previously treated NSCLC initiating subsequent-line therapy

- As of February 2016, we completed enrollment of 80 patients to Cohort 3 of our trial to allow serial assessment of plasma response and prediction of treatment outcome. These 80 patients, combined with 52 patients undergoing serial plasma draws from Cohorts 1 and 2, total the 132 patients for the follow-up analysis. Of these, 87 had both detectable mutations in plasma cfDNA as well as tumor imaging which was adequate for objective response analysis. We have completed the analysis of this cohort and are preparing the manuscript for publication.

iii. To optimize the sensitivity of cfDNA genotyping by studying factors impacting DNA quantity, quality, and purity

- Paired plasma specimens have been collected on 253 patients, allowing analysis of various handling conditions. The primary analyses were reported in the JAMA Oncology publication.

b. **What was accomplished under these goals?**

420 total patients have been enrolled to protocol DF/HCC protocol #14-147 as of 2/6/2017. This trial was initially launched with the support of this grant mechanism, and has since been amended to allow exploration of additional aims which are not directly related to or funded by this grant. The majority of patients on protocol #14-147 have been enrolled to the aims supported by this grant, as described in the table below:

Cohort	**Aim**	**Enrollment**	**DOD supported?**
1A	Accuracy of ddPCR in newly diagnosed advanced NSCLC	120 (complete)	Yes
1B	Exploration of ddPCR in early stage NSCLC	21	No
2A	Accuracy of ddPCR for acquired resistance to EGFR TKI	60 (complete)	Yes
2B	Exploration of ddPCR for study of drug resistance	9	No
3	Follow-up analysis of ddPCR for advanced NSCLC	80 (complete)	Yes
4	Exploration of plasma NGS technologies	130	No

The data from the three exploratory cohorts described above (1B, 2B, and 4) remains preliminary. We are working to generate data from early stage patients (Cohort 1B) that will allow prospective validation of ddPCR as a tool for early stage lung cancer through use of the NCI's ALCHEMIST trial (PI: Oxnard). Building off our pilot studies of plasma NGS (Cohort 4), we have now obtained funding from the Damon Runyon Cancer Research Foundation to allow a 200 patient prospective validation of plasma NGS versus ddPCR.

A total of 971 plasma specimens from 260 patients have undergone droplet digital PCR analysis for key EGFR and KRAS mutations in cohorts 1A, 2A, and 3. This methodology has been described previously in the grant application (Oxnard et al, CCR, 2014), but briefly involves emulsification of cfDNA into ~20,000 droplet for quantification of the absolute prevalence of mutant versus wildtype alleles (**Figure 1**).

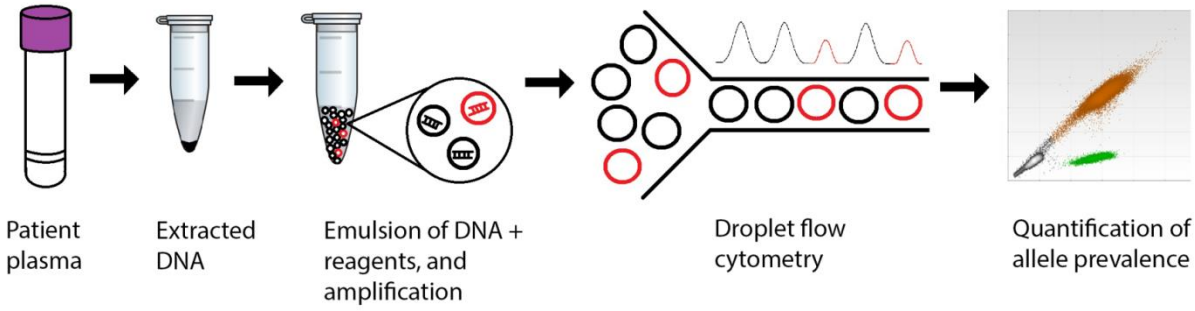

Figure 1: Plasma genotyping with ddPCR involves extracting cfDNA from plasma and making an emulsion of ~20,000 droplets. These are then run through a droplet flow cytometer where the proportion of positive and negative droplets is used to quantify the prevalence of a specific mutation.

We have successfully completed Aim 1, prospectively characterizing the sensitivity and specificity of our plasma ddPCR assay (**Figure 2**, top). Studying 115 cases with newly diagnosed or progressive NSCLC, specificity was 100% for the 3 driver mutations studied (EGFR L858R, EGFR exon 19 del, KRAS G12X). Sensitivity for these 3 assays was in the range of 64-86%, confirming that these assays do not have the 100% sensitivity needed to fully replace tumor biopsies for genotyping. For the T790M resistance mutation, 54 cases with acquired resistance were studied. Sensitivity was 77% but specificity was only 63% for the EGFR T790M resistance mutation, highlighting the complex heterogeneity of resistance. Sensitivity is improved in patients with a greater number of metastatic sites of disease (**Figure 2**, bottom). These data were published in JAMA Oncology, and are consistent with other retrospective plasma genotyping studies from our group (Oxnard et al, JCO, 2016).

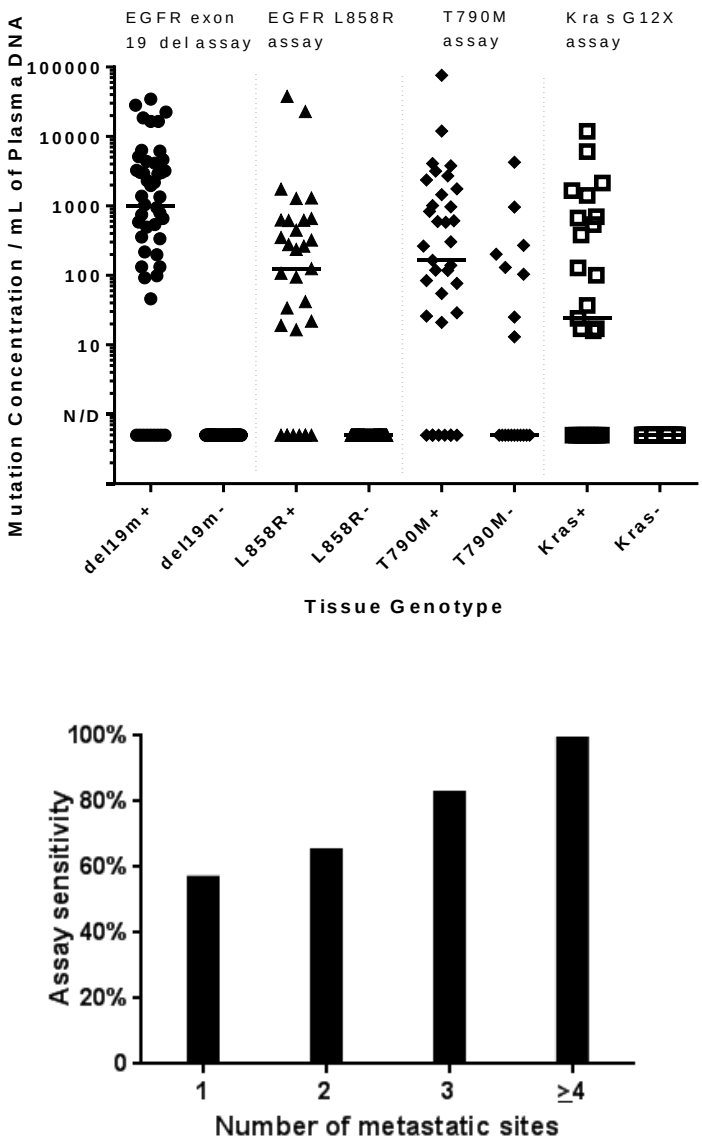

Figure 2: Dynamic range of plasma genotyping using a validated ddPCR-based assay. Wide dynamic range and the absence of false positives are noted for the detection of *KRAS* G12X and *EGFR* sensitizing mutations. A small number of false positives are seen with the *EGFR* T790M assay – potentially secondary to tumor heterogeneity with respect to acquired resistance mechanisms (n=174).

We have successfully completed Aim 3, studying paired plasma to understand the importance of various handling conditions (**Figure 3**). First, we compared standard rapid analysis of EDTA tubes to analysis after shipping tubes to our own lab overnight on ice. DNA concentration and ddPCR results were similar with both approaches. Second, we compared standard rapid analysis of EDTA tubes to rapid analysis of Streck tubes (more expensive) and found similar DNA concentration and ddPCR results. Lastly, we performed a paired ddPCR analysis in our own research lab with an analysis in our clinical pathology lab and found identical accuracy. This has led to the successful transition of our research assay into the pathology lab for clinical application, where it is being used for clinical trial enrollment.

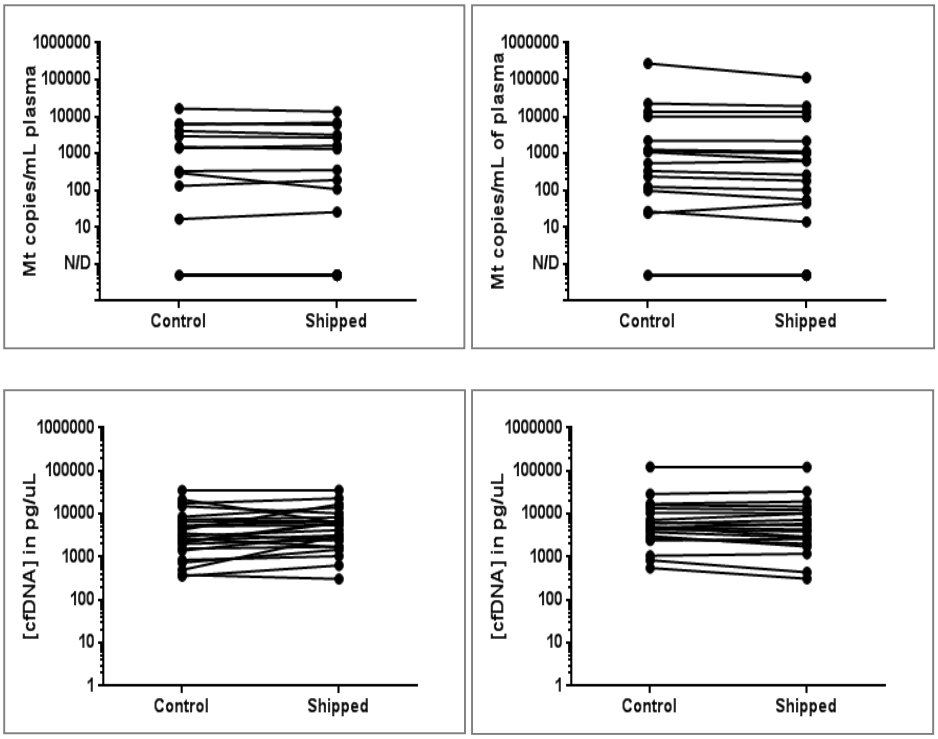

Figure 3: The mutant allele concentration (top) and total DNA (bottom) does not differ between mailed EDTA tube on ice (left) or mailed Streck tube at room temperature (right) when compared with a paired sample drawn simultaneously from the same patient and processed immediately as per standard operating procedure.

Finally, we have also completed our analysis of Aim 2 and are preparing this for publication. The results are less definitive as compared to the other aims, and lead to a range of additional questions that require study. As detailed above, 87 patients with detectable tumor mutations in cfDNA and measurable disease on tumor imaging were included. In an initial analysis, we found that plasma response kinetics are related to outcome (**Figure 4**).

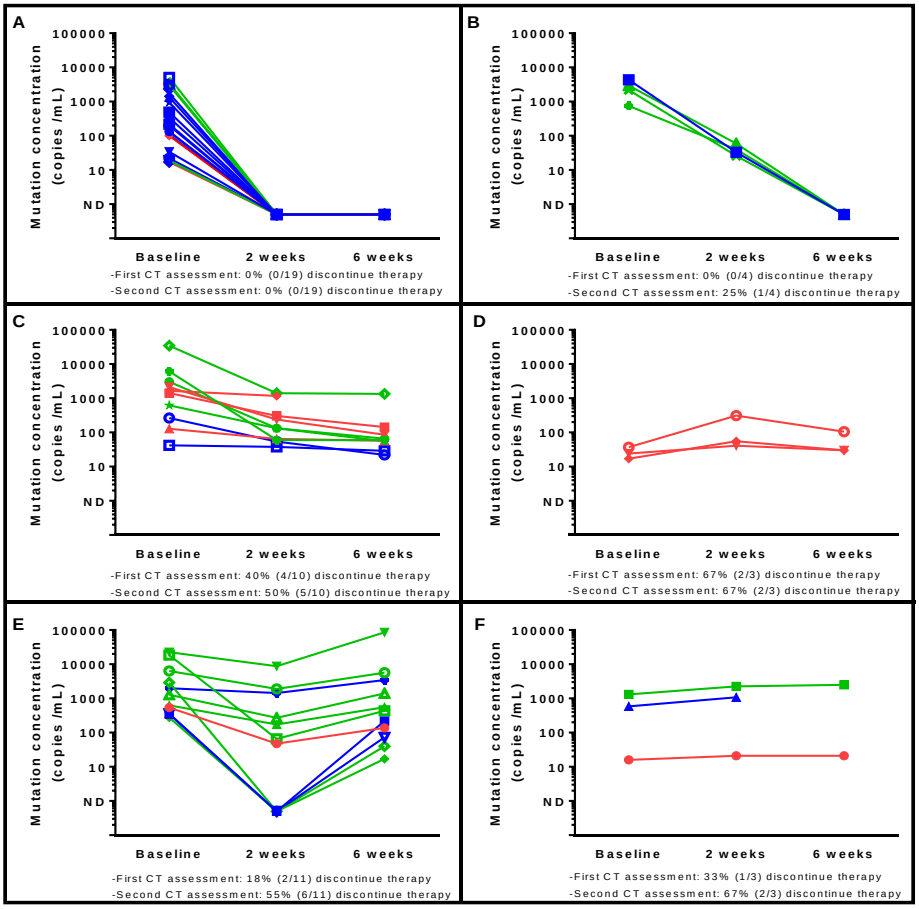

Figure 4: Patients with complete resolution of mutant cfDNA (A-B) exhibited a treatment discontinuation rate of 0% (0/23) and 4% (1/23) at initial and second restaging CT scans. Alternatively, patients without complete resolution (C-F) had a treatment discontinuation rate of 33% (9/27) at initial re-imaging and 56% (15/27) at second re-imaging assessment. Patient genotypes included EGFR sensitizing alone (blue), EGFR sensitizing in the presence of T790M (green) and KRAS G12X (red).

We have used tumor measurement to more directly study the relationship between pretreatment levels of plasma DNA and tumor diameter, and found them to be largely unrelated (**Figure 5**, left). This suggest shed of tumor DNA into plasma is a distinct biologic process, and not simply representative of tumor size. We then looked at response, comparing change in tumor diameter on treatment compared to early changes in mutation levels in plasma DNA after 2 weeks. This relationship was more complex than we had expected. Of 87 evaluable patients, 34 (39%) had a complete plasma response after 2 weeks of therapy (**Figure 5**, right), defined as undetectable levels of plasma mutations, and the median change in plasma levels was 99%. Patients with a >90% reduction in plasma mutation levels at 2 weeks had a 63% RECIST response rate on initial scans (34/54), while the remaining patients had only a 27% RECIST response rate (9/33, p=0.002).

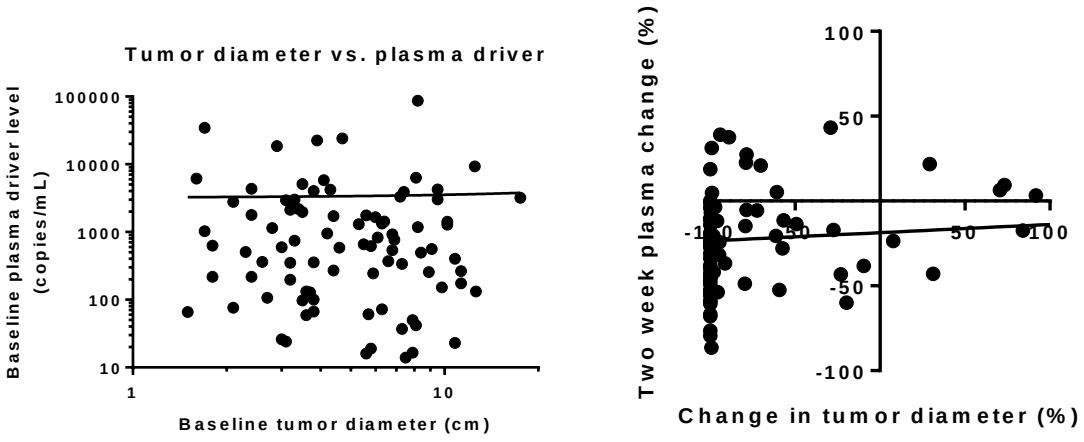

Figure 5: Little correlation is seen between baseline tumor diameter and level of driver mutation in plasma (left) for 87 patients with detectable EGFR exon 19 del or O858R, KRAS G12X, or BRAF V600E. Comparing change in diameter on initial scan with change in plasma levels at 2 weeks (right), a complex relationship is seen with a majority of patients having a dramatic reduction in plasma mutation levels, which predicts for a better response on imaging.

c. **What opportunities for training and professional development has the project provided?**

This project has had a transformative impact on my career development. The successful validation of this assay has created a large variety of opportunities for research and clinical application, as listed below. I have secured funding from the Damon Runyon Cancer Research Foundation and the Anna Fuller Fund to pursue these investigations, and have also recently submitted my first R01 application to the NIH.

1. Plasma genotyping for understanding drug resistance: I was senior/corresponding author of two publications in high impact journals (Thress et al, Nature Medicine 2015; Bahcall et al, Cancer Discovery, 2016) where we used plasma genotyping as a tool for detection of novel resistance mutations in NSCLC patients progressing on targeted therapy. I have presented these data at several international meetings (WCLC 2016, upcoming at AACR 2017.)

2. Plasma genotyping to guide clinical care: I was first/corresponding author of a publication in a high impact journal (Oxnard et al, JCO, 2016) describing the use of plasma T790M testing as an alternative to tumor T790M testing for treating EGFR inhibitor resistance in NSCLC. These data have led to presentations at international conferences (ELCC, ASCO) and regulatory meetings (AACR/FDA workshop). I secured institutional funding to launch our plasma assay as a clinical test at Brigham and Women's Hospital. With this assay, I have launched an investigator-sponsored trial (funding from Astellas) to study plasma genotyping for initiating treatment with erlotinib.

3. Plasma ddPCR as an orthogonal test for validating plasma NGS: Our leading ddPCR assay now serves as a benchmark for validation of newer assays. Using the specimens studied on this grant, we worked with a biotech startup to validate an approach that uses targeted NGS

of cfDNA, and published this successful assay development (Paweletz et al, CCR, 2016). We now continue to collaborating with leading plasma NGS companies (Guardant, Inivata) to assist in clinical validation their assays, hoping to facilitate regulatory development. Additional validation studies are expected to be presented this year (AACR and ASCO), with additional collaborations ongoing.

4. Plasma genotyping for evaluating drug effect in early stage clinical trials: We have received approval from the NCI Biomarker Review Committee (BRC) and have now integrated our plasma ddPCR assay into several ongoing CTEP trials (ETCTN). Each of these trials is studying combination targeted therapy for EGFR-mutant NSCLC. We have received funding from a UM1 supplement to perform plasma genotyping as a response marker to assist in measuring drug effect.

5. Plasma genotyping for detection of minimal residual disease after surgery: As Study Chair for the NCI's ALCHEMIST trial, I have had the opportunity to now integrate plasma genotyping into this study as a tool for detecting residual disease after surgery. We have received NCI approval of an amendment and are now starting to bank specimens from the trial. We hope to develop plasma genotyping as an independent staging tool for early stage NSCLC.

6. Plasma genotyping to identify incidental germline variants: One entirely unexpected result of our studies has been the finding that germline EGFR T790M mutations can be distinguished from somatic EGFR T790M mutations based on their allelic fraction in cfDNA. We are now collaborating with Guardant to query their database of plasma NGS

results in order to optimize bioinformatics strategies for incidental identification of germline cancer risk alleles within routine plasma NGS analysis.

- **How were the results disseminated to communities of interest?**

Effective implementation of plasma genotyping as a part of lung cancer care is a key message that I communicate in my educational talks at conferences, and are emphasized in the manuscript at JAMA Oncology.

2014	State-of-the-art Molecular Diagnosis and Targeted Therapy for Lung Cancer / Keynote Best of ASCO, Miami, FL
2014	Plasma genotyping as a tool for lung cancer care / BWH Clinical Pathology Conference Pathology Department, BWH, Boston, MA
2014	Personalizing treatment of resistance to EGFR TKI: what challenges? / Invited speaker Satellite symposium, European Society of Medical Oncology (ESMO) Annual Meeting 2014, Madrid, Spain (Sponsored by Astra-Zeneca)
2015	Novel definition of TKI resistance: Clinical versus molecular / Invited speaker European Lung Cancer Conference (ELCC), Geneva, Switzerland
2015	Replacing tumor biopsies with liquid biopsies: close but not quite / Invited discussant ASCO Annual Meeting, Chicago, IL
2016	Plasma Genotyping for Treatment Selection in Advanced Lung Cancer / Invited speaker FDA-AACR Liquid Biopsies in Oncology Drug and Device Development Workshop, Washington, DC
2016	Clinical applications of cell-free DNA genotyping for cancer care / Invited speaker Workshop of circulating tumor DNA (ctDNA) assays, National Cancer Institute, Bethesda, MD
2016	Plasma genotyping for predicting benefit from osimertinib in patients with advanced NSCLC / Oral presentation of submitted abstract European Lung Cancer Conference (ELCC), Geneva, Switzerland
2016	Validation and application of plasma genotyping for NSCLC / Invited speaker Japanese Lung Cancer Society, Fukuoka, Japan

Furthermore, the lay press expressed significant interest in our published data and assisted in the dissemination of this information to the public:

NBC Nightly News: http://www.nbcnews.com/video/liquid-biopsy-blood-test-could-help-fight-lung-cancer-study-661201475667
WHDH News: http://whdh.com/uncategorized/healthcast-new-blood-test-for-lung-cancer-treatment/

Huffington Post: http://www.huffingtonpost.com/bonnie-j-addario/a-blood-test-to-diagnose-_b_9994084.html
Tech Times: http://www.techtimes.com/articles/148392/20160409/liquid-biopsy-for-lung-cancer-simple-blood-test-can-detect-mutation-in-key-genes.htm
ScienceDaily: https://www.sciencedaily.com/releases/2016/04/160407115907.htm
Inquisitr: http://www.inquisitr.com/2984661/lung-cancer-early-detection-with-liquid-biopsy-blood-test/

- **What do you plan to do during the next reporting period to accomplish the goals?**

  Nothing to report

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

  Our approach, which was to develop a rapid noninvasive assay with high positive predictive value that generates actionable results, but with less than 100% sensitivity, has been well received by regulatory bodies (FDA) and pharmaceutical sponsors. This approach has been incorporated directly into the approval strategy for plasma genotyping assays for lung cancer care, which are intended to be used with a reflex to tumor genotyping if negative. Our group is well positioned to continue informing this narrative in the coming years

- **What was the impact on other disciplines?**

  Nothing to report

- **What was the impact on technology transfer?**

  Prior to the grant application, my team had submitted a patent application describing the approach we use for cfDNA genotyping using ddPCR. We have now licensed this expertise to one company to assist in the validation of their own liquid biopsy approach. We continue to work closely with other biotech companies and are interested in additional options for licensing our expertise.

- **What was the impact on society beyond science and technology?**

  Nothing to report

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

  The planned research has proceeded without significant change, except that the existing correlative study has been updated to permit additional correlative investigations funded through other avenues. In this way, the original protocol funded by this grant is likely to continue generating scientific opportunities for our group and for me as the awardee. The most significant change was the recent addition of a large cohort for paired plasma analysis using ddPCR and plasma NGS, towards the goal of validating a next-generation assay with our existing infrastructure and our now validated ddPCR assay

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  Nothing to report

- **Changes that had a significant impact on expenditures**

  Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations**
- **Journal publications.**

  1) Paweletz CP, Sacher AG, Raymond CK, Alden RS, O'Connell A, Mach SL, Kuang Y, Gandhi L, Kirschmeier P, English JM, Lim LP, Jänne PA, **Oxnard GR**. Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients. Clin Cancer Res 2016. Funding from Department of Defense was acknowledged.

  2) Sacher AG, Paweletz CP, Dahlberg SE, Alden RS, O'Connell A, Feeney N, Mach SL, Jänne PA, **Oxnard GR**. Prospective validation of rapid plasma genotyping as a sensitive and specific tool for guiding lung cancer care. JAMA Oncol 2016. Funding from Department of Defense was acknowledged.

- **Books or other non-periodical, one-time publications.**

  Nothing to report

- **Other publications, conference papers, and presentations.**

  Nothing to report

- **Website(s) or other Internet site(s)**

  Nothing to report

- **Technologies or techniques**

  Nothing to report

- **Inventions, patent applications, and/or licenses**

  Nothing to report

- **Other Products**

  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

  - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Name:	*Geoffrey Oxnard, MD– NO CHANGE*
Project Role:	*Assistant Professor of Medicine*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	
Funding Support:	

Name:	*Suzanne Dahlberg, PhD – NO CHANGE*
Project Role:	*Biostatistics, Senior Research Scientist*
Researcher Identifier (e.g. ORCID ID):	0000-0001-5139-831X
Nearest person month worked:	
Contribution to Project:	
Funding Support:	

Name:	*Ruthia Chen*
Project Role:	Research Data Specialist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4
Contribution to Project:	Assisted with screening patients for participation, patient consent, tracking of clinical data, ordering plasma collection, and tracking of specimens
Funding Support:	DOD grant (this project)

Name:	*Ryan Alden– NO CHANGE*
Project Role:	*Research Data Specialist*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	
Contribution to Project:	
Funding Support:	

Name:	*Stacy Mach*
Project Role:	Research Data Specialist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4
Contribution to Project:	Assisted with screening patients for participation, patient consent, tracking of clinical data, ordering plasma collection, and tracking of specimens
Funding Support:	DOD grant (this project)

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Annotated Other Support Document attached
- **What other organizations were involved as partners?**

  Nothing to report

- **SPECIAL REPORTING REQUIREMENTS**

  Not applicable

- **APPENDICES**

  - Annotated Other Support for Principal Investigator
  - Updated Biographical Sketch for Principal Investigator
  - Manuscript: Paweletz et al, CCR, 2016
  - Manuscript: Sacher et al, JAMA Oncol, 2016

OTHER SUPPORT

OXNARD, GEOFFREY R.

Active:

[NEW]
Foundation for the National Institute of Health (Oxnard) 01/13/17 – 01/12/19 0.84 CM

Biomarkers Consortium Vol-PACT: Advanced Metrics and Modeling with Volumetric CT for Precision Analysis of Clinical Trial Results
The project is aimed at improving the ability of randomized phase II trials to accurately predict phase III trial results. Proposal that more detailed assessment of tumor burden through use of volumetric CT measurement will improve the efficiency and accuracy of phase II trial analysis.
Role: Principal Investigator
Agency Contact: Stacey J. Adam, Ph.D., Scientific Program Manager

Damon Runyon Cancer Research Foundation (Oxnard) 07/01/16 – 06/30/19 1.20 CM

Nonivasive genotyping of cell free plasma DNA (cfDNA) as a tool for guiding personalized lung cancer care
Specific Aims: 1) Demonstrate the ability of plasma genomics to accelerate the delivery of highly active targeted therapies; 2) Validate the ability of plasma NGS to both detect complex targetable genotypes as well as distinguish underlying germline variants; 3) Study the intrapatient heterogeneity of resistance to targeted therapies using plasma genomics
Role: Principal Investigator
Agency Contact: Emily Turek, Programs Assistant

NIH/NCI P30 CA006516 (Glimcher) 03/01/16 – 02/28/18 1.80 CM
Supplement
2016 Cancer Clinical Investigator Team Leadership Award
The Cancer Clinical Investigator Team Leadership Award (CCITLA) is an administrative supplement award which recognizes and supports clinical investigators with an outstanding record of developing and promoting a culture of successful clinical research. It is the intent of the CCITLA to support mid-level clinical investigators at NCI designated Cancer Centers who are participating extensively in NCI-funded collaborative clinical trials and clinical research efforts.
Role: Principal Investigator – Team Leadership Award
Agency Contact: Jennifer Hayes, NCI CCCT CCITLA Program Director

R01 CA135257 (Jänne, P) 07/29/13 – 04/30/18 0.90 CM
NIH/NCI
Drug Resistance in Lung Cancer
The goal of the project is to study drug resistance mechanisms *in vitro* and using tumors from lung cancer patients with epidermal growth factor receptor (EGFR) mutations.
Role: Co-Investigator
Agency Contact: William C. Timmer, Program Official

R01 CA114465 (Johnson/Jänne) 07/09/12 – 04/30/17 0.90 CM NIH/ NCI

EGFR Mutations in Non-Small Cell Lung Cancer

The aims of the study are to prospectively validate the frequency and type of acquired resistance mutations and genomic changes arising in subjects with advanced NSCLC and somatic sensitizing mutations of *EGFR* treated with EGFR inhibitors.

Role: Co-Investigator

Agency Contact: Kelly Y. Kim, Program Official

*Anna Fuller Foundation (Oxnard) 10/01/15 – 12/31/16 1.20 CM

Expanded investigations into plasma genotyping for NSCLC using targeted NGS

Specific Aims: 1) We now propose to extend our study of plasma ddPCR and, using our existing infrastructure, additionally validate this new plasma NGS approach for advanced lung cancer; 2) Once validation is complete, we will initiate a prospective study that uses plasma NGS to guide treatment, without waiting for tumor genotype.

Role: Principal Investigator

Agency Contact: James Parker, Vice President

*Receiving a 1 year no cost extension

Overlap: All Active

None

Pending:

Research Project Grant (Parent R01) 09/01/17 – 08/31/22 1.32 CM NIH/NCI

Clinical Development of Plasma Genotyping as a Biomarker for the Management of Targeted Therapy Resistance in Lung Cancer

Specific Aims: 1) To demonstrate the power of remote plasma collection as a tool for understanding drug resistance in patients with rare NSCLC genotypes 2) To study the utility of serial plasma genotyping for detecting the emergence of competing resistance mechanisms in patients with genotype-driven NSCLC 3) To develop a point-of-care (POC) assay for real-time monitoring of response and resistance mechanisms in patients with inadequate plasma response to osimertinib

Role: PI

Agency Contact: Crystal Wolfrey, NCI

Completed:

*W81XWH-14-1-0128 (Oxnard) 10/01/14 – 09/30/16 2.40 CM Department of Defense

Personalization of lung cancer therapy using a new, clinical-grade assay for plasma-based measurement and monitoring of tumor genotype

This grant supports prospective validation of droplet digital PCR (ddPCR) as a new noninvasive and quantitative tool for measuring tumor genotyping in the plasma of advanced lung cancer patients. ddPCR will be studied both in newly diagnosed lung cancer patients as well as those with resistance to EGFR inhibitors

Role: Principle Investigator

Agency Contact: Elizabeth Yu, Science Officer, LCRP and TSCRP

Email: Elizabeth.l.yu2.ctr@mail.mil; Phone: (301) 619-8922

[Recently Completed]

Foundation for the National Institute of Health (Oxnard) 10/01/14 – 09/30/16 1.20 CM

Vol-PACT: Volumetric CT, Improving Metrics for Phase II

The project is aimed at developing trial-level biomarkers that can have a practical impact on oncology drug development, using an efficient data sharing approach that is itself staged carefully to provide a proof of concept at minimal cost and time before expanding to additional datasets and analyses.

Role: Principle Investigator
Agency Contact: Paul Eason, Scientific Program Manager

[Recently Completed]
Phi Beta Psi Charity Trust (Oxnard) 08/15/14 – 08/14/16 *****0.00 CM

Non-invasive genotyping realized at last: Personalization of cancer therapy using a new, clinical-grade assay for plasma-based measurement and monitoring of tumor genotype
Our overarching aim is to develop a plasma assay with 100% specificity and 100% positive predictive value (PPV), therefore allowing rapid adoption as a clinical biomarker.
Role: Principal Investigator
Agency Contact: Paula K. Dunn, National Project Chairperson

*****Sponsor guidelines prohibit salary from being used for PI salary support.

Conquer Cancer Foundation of ASCO (Oxnard) 07/01/12 – 06/30/15 1.50 CM
Career Development Award
Characterizing a new familial lung cancer syndrome through the identification and study of patients with germline EGFR mutations
In the proposed study, we use this relationship between somatic and germline T790M to identify patients carrying germline EGFR mutations, for accrual to a registry for the prospective study of the natural history of this condition.
Role: Principal Investigator

MO Infrastructure Award (Oxnard) 10/01/13 – 06/30/15 0.30 CM
DFCI - Internal
Infrastructure for development and clinical implementation of plasma-based tumor genotyping across disease centers at the Dana-Farber / Brigham and Women's Cancer Center
With this infrastructure grant, we will broaden assay development into other cancer types and into routine clinical use.
Role: Principal Investigator
Agency Contact: David Read, Vice President of Ambulatory Care

Bonnie J. Addario Lung Cancer Foundation (Oxnard) 01/01/13 – 12/31/14 3.00 CM

Characterizing a new familial lung cancer syndrome through the identification and study of patients with germline EGFR mutations
This project will establish a multi-centered trial for the study of patients carrying germline EGFR mutations in order to characterize lung cancer risk through CT screening.
Role: Principal Investigator
Agency Contact: Scott Santarella, President and CEO

Wong Family Award (Oxnard0 09/01/13 – 08/30/14 0.60 cm
Dana-Farber Cancer Institute
Validating targeted next-generation sequencing with OncoPanel for the detection of targetable gene rearrangements and gene amplifications in non-small cell lung cancer
This biomarker development project will study a cohort of lung cancers known to be positive (ALK rearranged, ROS1 rearranged, etc.) and negative (KRAS-mutant) for targetable rearrangements and amplifications to allow next-generation sequencing to replace FISH as a gold-standard for detection.
Role: Principal Investigator

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: **Oxnard, Geoffrey R.**

eRA COMMONS USER NAME (credential, e.g., agency login): OXNARDGMSKCC

POSITION TITLE: **Assistant Professor of Medicine**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Harvard University, MA	BA	06/1999	Chemistry
University of Chicago, Pritzker School of Medicine, IL	MD	06/2005	Medicine
Massachusetts General Hospital, MA	Residency	06/2008	Internal Medicine
Memorial Sloan-Kettering Cancer Center, NY	Fellowship	02/2011	Medical Oncology

A. Personal Statement.

I am a thoracic oncologist at Dana Farber Cancer Institute where I perform translational research to identify better biomarkers and therapies for lung cancer. I am an early stage investigator (ESI), having completed medical training within the past 10 years and not previously received NIH funding. My prior studies have focused on genotype-defined subtypes of lung cancer, such as *EGFR*-mutant lung cancer, and understanding clinical biology of drug sensitivity and resistance. More recently, I have focused on application of plasma genotyping of cell-free DNA as a tool for detecting response to targeted therapy and eventual emergence of resistance, as well as for non-invasive genomic characterization of resistance mechanisms. The proposed project, to advance innovative applications of plasma genotyping for lung cancer care, was personally conceived by me and I will oversee the project's implementation. I will be responsible for implementation of the proposed clinical studies and coordinating the proposed collaborations between our clinical group (Lowe Center for Thoracic Oncology), our local laboratories (Belfer Center at DFCI and Center for Advanced Molecular Diagnostics at BWH), and our established biotech collaborators (Resolution Bioscience and Bio-Rad).

1. Sacher AG, Paweletz CP, Dahlberg SE, Alden RS, O'Connell A, Feeney N, Mach SL, Jänne PA, **Oxnard GR**. Prospective validation of rapid plasma genotyping as a sensitive and specific tool for guiding lung cancer care. JAMA Oncol 2016; PMID: 27055085
2. Paweletz CP, Sacher AG, Raymond CK, Alden RS, O'Connell A, Mach SL, Kuang Y, Gandhi L, Kirschmeier P, English JM, Lim LP, Jänne PA, **Oxnard GR**. Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients. Clin Cancer Res 2016; 22:915-22. PMCID: PMC4755822 [Available on 2017-02-15]
3. **Oxnard GR**, Thress KS, Alden RA, Lawrence R, Paweletz CP, Cantarini M, Yang JC, Barrett JC, Jänne PA. Association between Plasma Genotyping and Outcomes of Treatment with Osimertinib (AZD9291) in Advanced NSCLC. J Clin Oncol 2016. PMCID: PMC5035123 [Available on 2017-10-01]
4. Bahcall M, Sim T, Paweletz CP, Patel JD, Alden RS, Kuang Y, Sacher AG, Kim ND, Lydon CA, Awad MA, Jaklitsch MT, Sholl LM, Jänne PA*, **Oxnard GR***. Acquired *MET* D1228V mutation and resistance to MET inhibition in lung cancer. Cancer Discovery. PMCID: PMC5140694 [Available on 2017-12-01]

B. Positions and Honors.

Positions and Employment:

2005-2008 **Internal Medicine Residency**, Massachusetts General Hospital, Boston, MA
2008-2011 **Medical Oncology Fellow**, Memorial Sloan-Kettering Cancer Center, New York, NY
2011-2013 **Instructor in Medicine**, Harvard Medical School, Boston, MA

2011- **Attending Physician**, Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA
2013- **Assistant Professor of Medicine**, Harvard Medical School, Boston, MA

Extramural Activities:

2010- Alliance / CALGB Imaging Committee, Cadre Member
2011- Alliance / CALGB Respiratory Committee, Cadre Member
2011- Ad hoc reviewer: *New England Journal of Medicine, Nature Medicine, Journal of the American Medical Association (JAMA), Journal of Clinical Oncology, Journal of the National Cancer Institute*, *JAMA Oncology, Clinical Cancer Research, Cancer, Annals of Oncology, Clinical Pharmacology & Therapeutics, Journal of Thoracic Oncology, Journal of Molecular Diagnostics*

Honors:

1999 *Magna Cum Laude* in Chemistry, Harvard University
2004 AOA (Alpha Omega Alpha) Honor Medical Society, Pritzker School of Medicine
2004 Calvin Fentress Research Fellowship, Pritzker School of Medicine
2005 Graduation with Honors, Pritzker School of Medicine
2005 Franklin McLean Medical Student Research Award, Pritzker School of Medicine
2005 Pritzker Leadership Award, Pritzker School of Medicine
2005 Departmental Award, Department of Pathology, Pritzker School of Medicine
2010 Young Investigator Award, Conquer Cancer Foundation of ASCO
2010 Loan Repayment Program, National Institute of Health
2010, 2011 Merit Award, Conquer Cancer Foundation, ASCO Annual Meeting, Chicago
2011 Aspen AACR Workshop: Molecular Biology in Clinical Oncology
2012 Career Development Award, Conquer Cancer Foundation of ASCO
2013 Young Investigator Travel Award, 15th IASLC World Conference on Lung Cancer, Sydney
2013 Ellen and Steven Fine Teaching Award, Dana-Farber Cancer Institute
2014 Ascelpios Award, Bonnie J. Addario Lung Cancer Foundation
2016 Cancer Clinical Investigator Team Leadership Award, NCI

C. Contribution to Science.

1. Using plasma genotyping to characterize response and resistance to targeted therapies: Given the key role of tumor genotyping in lung cancer care and research, my recent research has focused on developing plasma genotyping of cell-free DNA. I led the clinical development and prospective validation of an internally developed droplet digital PCR (ddPCR) assay, which has now been launched for clinical use at our institution. We have used plasma genotyping to describe the discovery of acquired EGFR C797S resistance mutations after treatment with osimertinib, and acquired MET D1228V mutations after treatment with savolitinib. We developed and clinically piloted a new assay for targeted next-generation sequencing (NGS) of plasma cfDNA from advanced lung cancer patients, which can rapidly detect all types of targetable oncogenic alterations and resistance mechanisms in cfDNA. I serve as primary investigator for all of these studies.
    a. Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, Lai Z, Markovets A, Vivancos A, Kuang Y, Ercan D, Matthews SE, Cantarini M, Barrett JC, Jänne PA, **Oxnard GR**. Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. Nat Med 2015; 21:560-2. PMCID: PMC4771182.
    b. Paweletz CP, Sacher AG, Raymond CK, Alden RS, O'Connell A, Mach SL, Kuang Y, Gandhi L, Kirschmeier P, English JM, Lim LP, Jänne PA, **Oxnard GR**. Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients. Clin Cancer Res 2016; 22:915-22. PMCID: PMC4755822.
    c. Sacher AG, Paweletz CP, Dahlberg SE, Alden RS, O'Connell A, Feeney N, Mach SL, Jänne PA, **Oxnard GR**. Prospective validation of rapid plasma genotyping as a sensitive and specific tool for guiding lung cancer care. JAMA Oncol 2016; Epub ahead of print.
    d. Bahcall M, Sim T, Paweletz CP, Patel JD, Alden RS, Kuang Y, Sacher AG, Kim ND, Lydon CA, Awad MA, Jaklitsch MT, Sholl LM, Jänne PA*, **Oxnard GR***. Acquired *MET* D1228V mutation and resistance to MET inhibition in lung cancer. Cancer Discovery. PMCID: PMC5140694 [Available on 2017-12-01]

2. Understanding and targeting acquired resistance to EGFR inhibitors in lung cancer: For 8 years I have worked at the intersection of preclinical scientists, molecular pathologists, and clinical investigators to study the biology of the EGFR T790M resistance mutation in lung cancer patients. In a series of publications, collaborators and I established a unique indolent biology to T790M-mediated resistance that lends a more favorable prognosis and allows clinicians to delay change of therapy in the setting of asymptomatic progression. Of 12 research articles co-authored on this topic, I served as lead author or primary investigator on 6.
    a. **Oxnard GR**, Arcila ME, Sima CS, Riely GJ, Chmielecki J, Kris MG, Pao W, Ladanyi M, Miller VA. 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. PMCID: PMC3060283
    b. **Oxnard GR**, Janjigian YY, Arcila ME, Sima CS, Kass SL, Riely GJ, Pao W, Kris MG, Ladanyi M, Azzoli CG, Miller VA. Maintained sensitivity to EGFR tyrosine kinase inhibitors in EGFR-mutant lung cancer recurring after adjuvant erlotinib or gefitinib. Clin Cancer Res 2011; 17:6322-8. PMCID: PMC3186869
    c. Lo PC, Dahlberg SE, Nishino M, Johnson BE, Sequist LV, Jackman DM, Janne PA, **Oxnard GR**. Delay of treatment change following objective progression on first-line erlotinib in EGFR-mutant lung cancer. Cancer 2015; 121:2570-7.
    d. **Oxnard GR**, Thress KS, Alden RA, Lawrence R, Paweletz CP, Cantarini M, Yang JC, Barrett JC, Jänne PA. Association between Plasma Genotyping and Outcomes of Treatment with Osimertinib (AZD9291) in Advanced NSCLC. J Clin Oncol 2016; PMCID: PMC5035123 [Available on 2017-10-01]

3. Clinical characteristics and outcomes associated with rare genotypes of NSCLC: My studies of acquired EGFR T790M has also led to study of other rare EGFR mutations found in NSCLC, focusing first on EGFR T790M occurring at lung cancer diagnosis, which we found to be associated with an underlying germline T790M mutation. This work resulted in my leading the first prospective trial to characterize this rare but important familial lung cancer syndrome. Additional investigations have studied insertion mutations in EGFR exon 19 and exon 20, some of which can be sensitive to EGFR targeted therapy. Lastly, I have identified an enrichment for rare lung cancer genotypes in patients with lung cancer diagnosed at a young age (<50). Together these investigations leave me with a broad understanding of the molecular biology of oncogenic mutations in NSCLC and interest in improving the care of individual rare lung cancer subtypes. I served as primary investigator for all of these studies.
    a. **Oxnard GR**, Miller VA, Robson ME, Azzoli CG, Pao W, Ladanyi M, Arcila ME. Screening for germline EGFR T790M mutations through lung cancer genotyping. J Thorac Oncol 2012; 7:1049-52. PMCID: PMC3354706
    b. He M, Capelletti M, Nafa K, Yun CH, Arcila ME, Miller VA, Ginsberg MS, Zhao B, Kris MG, Eck MJ, Jänne PA, Ladanyi M, **Oxnard GR**. *EGFR* Exon 19 Insertions: A New Family of Sensitizing *EGFR* Mutations in Lung Adenocarcinoma. Clin Cancer Res; 2012; 18:1790-7. PMCID: PMC3306520
    c. **Oxnard GR**, Lo P, Nishino M, Dahlberg S, Lindeman NI, Butaney M, Jackman DM, Johnson BE, Jänne PA. Natural history and molecular characteristics of lung cancers harboring EGFR exon 20 insertions. J Thorac Oncol 2013; 8: 179-84. PMCID: PMC3549533
    d. Sacher AG, Dahlberg SE, Heng J, Mach S, Jänne PA, **Oxnard GR**. Association between younger age and targetable genomic alterations and prognosis in non-small cell lung cancer. JAMA Oncol 2016;2:313-20. PMCID: PMC4819418

4. Improving CT-based tumor measurements as a tool for drug development: Optimizing CT-based tumor measurement for thoracic cancers has been a research interest of mine since working in an imaging lab in medical school. Since then, I have worked closely with Dr. Lawrence Schwartz of Columbia University to optimize CT-based tumor measurement as a tool for guiding clinical care and translational research. In ongoing work, we are studying objective response rate and its relationship to regulatory approval in single arm studies, and are working with the FNIH to re-analyze images from landmark randomized trials toward developing better imaging endpoints. I serve as lead investigator for all of these studies.

a. **Oxnard GR**, Zhao BZ, Sima CS, Ginsberg MS, James LP, Lefkowitz RA, Guo P, Kris MG, Schwartz LH, Riely GJ. Variability of lung tumor measurements on computed tomography (CT) scans taken within 15 minutes. J Clin Oncol 2011; 29:3114-9. PMCID:PMC3157977
b. Zhao BZ, **Oxnard GR**, Moskowitz CS, Kris MG, Pao W, Guo P, Rusch VM, Ladanyi M, Rizvi NA, Schwartz LH. A pilot study of volume measurement as a method of tumor response evaluation to aid biomarker development. Clin Cancer Res 2010; 16:4647-53. PMCID:PMC2940965
c. **Oxnard GR**, Wilcox KH, Gonen M, Polotsky M, Hirsch BR, Schwartz LH. Response rate as a regulatory endpoint in single-arm studies of advanced solid tumors. JAMA Oncol 2016; 2:772-9.

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/myncbi/browse/collection/40210808/?sort=date&direction=descending

D. Research Support.

Ongoing:

Damon Runyon Cancer Research Foundation (Oxnard) 07/01/16 – 06/30/19
Nonivasive genotyping of cell free plasma DNA (cfDNA) as a tool for guiding personalized lung cancer care
Specific Aims: 1) Demonstrate the ability of plasma genomics to accelerate the delivery of highly active targeted therapies; 2) Validate the ability of plasma NGS to both detect complex targetable genotypes as well as distinguish underlying germline variants; 3) Study the intrapatient heterogeneity of resistance to targeted therapies using plasma genomics
Role: Principal Investigator

NIH/NCI R01 CA135257 (Jänne) 07/01/13 – 06/30/18
Drug Resistance in Lung Cancer
The goal of the project is to study drug resistance mechanisms *in vitro* and using tumors from lung cancer patients with epidermal growth factor receptor (EGFR) mutations.
Role: Co-Investigator

NIH/NCI P30 CA006516 (Glimcher) 03/01/16 – 02/28/18
Supplement
2016 Cancer Clinical Investigator Team Leadership Award
The Cancer Clinical Investigator Team Leadership Award (CCITLA) is an administrative supplement award which recognizes and supports clinical investigators with an outstanding record of developing and promoting a culture of successful clinical research. It is the intent of the CCITLA to support mid-level clinical investigators at NCI designated Cancer Centers who are participating extensively in NCI-funded collaborative clinical trials and clinical research efforts.
Role: Principal Investigator – Team Leadership Award

NIH/NCI R01 CA114465 (Johnson/Jänne) 07/09/12 – 04/30/17
EGFR Mutations in Non-Small Cell Lung Cancer
The aims of the study are to prospectively validate the frequency and type of acquired resistance mutations and genomic changes arising in subjects with advanced NSCLC and somatic sensitizing mutations of *EGFR* treated with EGFR inhibitors.
Role: Co-Investigator

Foundation for the National Institute of Health (Oxnard) 01/13/17 – 01/12/19
Biomarkers Consortium Vol-PACT: Advanced Metrics and Modeling with Volumetric CT for Precision Analysis of Clinical Trial Results
The project is aimed at improving the ability of randomized phase II trials to accurately predict phase III trial results. Proposal that more detailed assessment of tumor burden through use of volumetric CT measurement will improve the efficiency and accuracy of phase II trial analysis.
Role: Principal Investigator

*Anna Fuller Foundation (Oxnard) 10/01/15 – 12/31/16

Expanded investigations into plasma genotyping for NSCLC using targeted NGS

Specific Aims: 1) We now propose to extend our study of plasma ddPCR and, using our existing infrastructure, additionally validate this new plasma NGS approach for advanced lung cancer; 2) Once validation is complete, we will initiate a prospective study that uses plasma NGS to guide treatment, without waiting for tumor genotype.

Role: Principal Investigator

*Receiving a 1 year no cost extension

Completed: Last three years

LCRP Career Development Award, Department of Defense (Oxnard) 10/01/14 – 09/30/16

Personalization of lung cancer therapy using a new, clinical-grade assay for plasma-based measurement and monitoring of tumor genotype

This grant supports a prospective trial to validate droplet digital PCR as an assay for personalization of lung cancer care, specifically studying it for initial genotyping, resistance genotyping, and response monitoring.

Role: Principal Investigator

Foundation for the National Institute of Health (Oxnard) 10/01/14 – 09/30/16

Vol-PACT: Volumetric CT, Improving Metrics for Phase II

The project is aimed at developing trial-level biomarkers that can have a practical impact on oncology drug development, using an efficient data sharing approach that is itself staged carefully to provide a proof of concept at minimal cost and time before expanding to additional datasets and analyses.

Role: Principal Investigator

Phi Beta Psi Charity Trust (Oxnard) 08/15/14 – 08/14/16

Non-invasive genotyping realized at last: Personalization of cancer therapy using a new, clinical-grade assay for plasma-based measurement and monitoring of tumor genotype

Our overarching aim is to develop a plasma assay with 100% specificity and 100% positive predictive value (PPV), therefore allowing rapid adoption as a clinical biomarker.

Role: Principal Investigator

Conquer Cancer Foundation of ASCO (Oxnard) 07/01/12 – 06/30/15

Characterizing a new familial lung cancer syndrome through the identification and study of patients with germline EGFR T790M mutations

This study examines the relationship between somatic and germline T790M in cancer patients and establishes the groundwork for a comprehensive study of inherited EGFR mutations and lung cancer risk.

Role: Principal Investigator

Bonnie J. Addario Lung Cancer Foundation (Oxnard) 01/01/13 – 12/31/14

Characterizing a new familial lung cancer syndrome through the identification and study of patients with germline EGFR mutations

This project will establish a multi-centered trial for the study of patients carrying germline EGFR mutations in order to characterize lung cancer risk through CT screening.

Role: Principal Investigator

Wong Family Award (Oxnard) 09/01/13 – 08/30/14

Dana-Farber Cancer Institute

Validating targeted next-generation sequencing with OncoPanel for the detection of targetable gene rearrangements and gene amplifications in non-small cell lung cancer

This biomarker development project will study a cohort of lung cancers known to be positive (ALK rearranged, ROS1 rearranged, etc.) and negative (KRAS-mutant) for targetable rearrangements and amplifications to allow next-generation sequencing to replace FISH as a gold-standard for detection.

Role: Principal Investigator

HHS Public Access

Author manuscript

Clin Cancer Res. Author manuscript; available in PMC 2017 February 15.

Published in final edited form as:

Clin Cancer Res. 2016 February 15; 22(4): 915–922. doi:10.1158/1078-0432.CCR-15-1627-T.

Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients

Cloud P. Paweletz[1], **Adrian G. Sacher**[2], **Chris K. Raymond**[3], **Ryan S. Alden**[2], **Allison O'Connell**[1], **Stacy L. Mach**[2], **Yanan Kuang**[1], **Leena Gandhi**[2], **Paul Kirschmeier**[1], **Jessie M. English**[1], **Lee P. Lim**[3], **Pasi A. Jänne**[1,2], and **Geoffrey R. Oxnard**[2,*]

[1]Belfer Center for Applied Cancer Science, Dana-Farber Cancer Institute, Boston MA

[2]Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston MA

[3]Resolution Bioscience, Bellevue, WA

Abstract

Purpose—Tumor genotyping is a powerful tool for guiding non-small cell lung cancer (NSCLC) care, however comprehensive tumor genotyping can be logistically cumbersome. To facilitate genotyping, we developed a next-generation sequencing (NGS) assay using a desktop sequencer to detect actionable mutations and rearrangements in cell-free plasma DNA (cfDNA).

Experimental Design—An NGS panel was developed targeting 11 driver oncogenes found in NSCLC. Targeted NGS was performed using a novel methodology that maximizes on-target reads, and minimizes artifact, and was validated on DNA dilutions derived from cell lines. Plasma NGS was then blindly performed on 48 patients with advanced, progressive NSCLC and a known tumor genotype, and explored in two patients with incomplete tumor genotyping.

Results—NGS could identify mutations present in DNA dilutions at ≥0.4% allelic frequency with 100% sensitivity/specificity. Plasma NGS detected a broad range of driver and resistance mutations, including *ALK*, *ROS1*, and *RET* rearrangements, *HER2* insertions, and *MET* amplification, with 100% specificity. Sensitivity was 77% across 62 known driver and resistance mutations from the 48 cases; in 29 cases with common *EGFR* and *KRAS* mutations, sensitivity was similar to droplet digital PCR. In two cases with incomplete tumor genotyping, plasma NGS rapidly identified a novel *EGFR* exon 19 deletion and a missed case of *MET* amplification.

Conclusion—Blinded to tumor genotype, this plasma NGS approach detected a broad range of targetable genomic alterations in NSCLC with no false positives including complex mutations like rearrangements and unexpected resistance mutations such as *EGFR* C797S. Through use of widely available vacutainers and a desktop sequencing platform, this assay has the potential to be implemented broadly for patient care and translational research.

*Corresponding Author: Geoffrey R. Oxnard, MD, Dana-Farber Cancer Institute, 450 Brookline Ave, Boston, MA, Ph: 617-632-6049, F: 617-632-5786, geoffrey_oxnard@dfci.harvard.edu.

Prior presentation: A portion of this data was presented previously as an oral presentation at the AACR Annual Meeting 2015

All remaining authors have no conflicts of interest.

Introduction

Genotype-directed targeted therapies are revolutionizing cancer care. Genomic alterations in genes such as *EGFR*, *ALK*, *KRAS*, and *BRAF* have been validated as powerful predictive biomarkers in the management of non-small cell lung cancer (NSCLC), colorectal cancer, and melanoma; it is now standard to test for these mutations to personalize treatment decisions.(1-7) Development of new genotype-directed therapies is widespread in solid tumor oncology, leading to increasing application of next-generation sequencing (NGS) panels that can test tumor biopsies for a wide range of potentially targetable mutations.(8, 9) However, routine use of NGS for tumor genotyping presents practical challenges including the availability of adequate biopsy specimens, slow turnaround time, and the need for repeat biopsies after development of drug resistance.(9) Given these challenges, it is clear that there is an unmet need for noninvasive assays that can broadly detect actionable genomic alterations.

Many groups, including our own, have investigated noninvasive tumor genotyping of cell-free plasma DNA (cfDNA) as an alternative to tissue genotyping.(10-15) Rather than studying circulating cells, these technologies study the free floating DNA contained in the plasma; in advanced cancer patients, a portion of this cfDNA may be derived from the tumor. Plasma genotyping has the potential to be less invasive and faster than tumor genotyping, while also allowing serial assessment of genotype during development of treatment resistance. We recently reported on a highly specific and rapid droplet digital PCR (ddPCR) assay for quantifying the concentration of *EGFR* and *KRAS* mutations in cfDNA of advanced NSCLC patients.(16, 17) Such PCR-based plasma assays test for mutations at a single site in a gene, but are limited by their inability to detect more complex genomic alterations such as chromosomal rearrangements and their inability to multiplex across several genes. Others have studied NGS of cfDNA using PCR amplicons or tagged DNA baits to enrich for target DNA sequences; however, many such assays are unable to detect rearrangements, while other assays rely on massive sequencing and computational processing resulting in unacceptable costs and slow turnaround time.(11, 18) While detection of mutant cfDNA present at low concentration is possible with these approaches despite the more abundant wildtype (germline) DNA, a universal challenge with these highly sensitive genotyping assays is the risk of false positives due to PCR artifact.

In this study, we piloted a novel targeted NGS approach for the detection of driver mutations and rearrangements in cfDNA from advanced NSCLC patients. Taking cues from traditional hybrid capture approaches that isolate genomic subsets by pull down with probes to genes of interest, our methodology improves on key steps during library generation to reduce sequencing demands and turnaround times. First, to maximize on-target reads to ∼90%, a two-step pull-down process was used that includes both a thermodynamically-controlled hybridization step and a kinetically-controlled extension step under conditions that neutralize GC bias. Then, to improve signal-to-noise ratio, tags were connected to each captured DNA fragment, so that every read is anchored to its clonal family and to its pull-down probe of origin, facilitating identification of low frequency mutant alleles and quantification of subtle changes in gene copy numbers (Fig. 1, Supplemental Methods). We hypothesized that this approach would allow for accurate detection of a broad range of

targetable genotypes, including insertions/deletions and rearrangements, in cfDNA from advanced NSCLC patients. Our goal was to leverage a desktop sequencing platform to enable a rapid turnaround time and facilitate widespread clinical adoption.

Methods

Plasma next-generation sequencing

Targeted NGS of cell-line DNA and plasma cfDNA was performed at Resolution Bioscience (Bellevue, WA) as described in Supplemental Methods. Chimeric gene fusions were detected using tiled probes that allow sequencing-based discovery of *de novo* rearrangements (Supplemental Fig 1 and Supplemental Table 1).

Plasma ddPCR

For comparison to plasma NGS, plasma ddPCR was performed using an established and validated assay which has been described previously.(16) Briefly, this assay emulsifies extracted plasma cfDNA into thousands of droplets which subsequently undergo individual PCR with custom fluorescently labeled probes designed to detect *EGFR* L858R, *EGFR* exon 19 deletions, or *KRAS* G12X.(16) Individual droplets are then read in a flow cytometer and the number of positive droplets are quantified (BioRad). Each sample is analyzed in triplicate.

Cell line validation

The targeted NGS panel was validated using genomic DNA from 14 independent, genetically-annotated cell lines harboring four gene fusions, 19 point mutations and two insertions/deletions (Supplemental Table 2). Cell lines were combined into two separate DNA pools, each containing the genomes of 7 cell lines, and systematically blended with normal, wild-type DNA to produce admixtures at 2.5%, 1.0%, 0.4% and 0.1% dilutions. Prior to NGS, DNA pools were acoustically fragmented to an average size distribution centered around 165 bp and purified by two-sided SPRI to give fragment profiles of 150-200 bp that closely approximate cfDNA. Cell lines for the analytical validation experiment were obtained from ATCC (A549, H2228, SK-MEL-2, H1666, SW48), RIKEN (Lc-2/ad), the Broad institute (H1781, SW480, HCT116, H2347, HCC78) and the NCI (H3122). PC-9 and H1975 cells were obtained from the laboratory of Dr. Pasi Jänne. All cell lines were validated to be correct by short tandem repeat analyses (STR).

Patient population

Patients were identified during their routine lung cancer care at Dana-Farber Cancer Institute. Patients were deemed eligible if they had advanced NSCLC with a known tumor genotype, either untreated or progressive on therapy. Tumor genotyping was performed as part of routine care, either using conventional genotyping assays (PCR, FISH) or a targeted NGS panel when available.(5, 19) All patients consented to plasma collection and analysis on an IRB-approved prospective plasma collection protocol or associated correlative science protocols. Following clinical validation, plasma NGS was explored in two patients with high suspicion of a targetable genotype missed on tumor genotyping.

Plasma collection

Plasma was collected prior to initiation of therapy for untreated or progressive advanced NSCLC. Whole blood was collected into 10 mL EDTA containing “purple-top” vacutainer tubes, centrifuged for 10 min at 1200g and the plasma supernatant was further cleared by centrifugation for 10 min at 3000g. Cleared plasma was stored in cryostat tubes at -80C until use. cfDNA was isolated using the QIAmp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's protocol. DNA was eluted in AVE buffer (100 uL) and stored at -80C until use.

Blinding of specimens

To ensure data integrity in these experiments, the samples were identified only by sample key. Only the clinical team (GRO, AGS, RSA) that identified patients for study had access to the tissue genotype results. The teams involved in ddPCR and NGS analysis were blinded during data acquisition (plasma isolation, cfDNA extraction, library generation, NGS and ddPCR analysis) and unblinding was done after NGS and ddPCR results had been reported to the clinical team.

Results

A probe set was developed that covers portions of eleven genes known to be targetable oncogenic drivers in NSCLC. Selected coding regions of eight genes were sequenced (*KRAS*, *EGFR*, *ALK*, *HER2*, *BRAF*, *NRAS*, *PIK3CA*, *MET*, and *MEK1*). Additionally, intronic probes were designed to detect genome level rearrangements that create chimeric gene fusions in *ALK*, *ROS1*, and *RET*. The coding regions of the tumor suppressor *TP53* were also included as a control because this gene is commonly mutated in NSCLC. Analysis of the performance of this probe set on plasma DNA showed >80% on-target percentage, which compares favorably with the less than 50% on-target percentage seen in previously published data using standard hybridization selection on plasma DNA (Supplementary Fig. 2).(18)

The targeted NGS panel was initially validated with dilutions of cell line DNA. Four different dilutions of two DNA pools were sequenced, each derived from 7 cell lines harboring previously characterized mutations (Supplemental Table 2). Dilutions of 2.5% to 0.1% resulted in calculated allelic frequencies ranging from 4.5% to 0.01% (Supplemental Fig. 3). Variant calling algorithms (Supplemental Methods) were able to identify mutations that were present at 0.1% or greater with sensitivity and specificity of 88% and 100%. Diagnostic performance improved to 100% sensitivity and specificity for mutations present at an allelic frequency 0.4% or greater (Supplemental Fig. 3, Supplemental Table 2). This preliminary analysis of the cell lines allowed the setting of thresholds that ensured high specificity in the subsequent analysis of patient samples.

After validating the NGS platform with DNA dilutions, plasma samples from 48 patients with advanced NSCLC were studied, blinded to the tumor genotyping results (Supplemental Table 3). The median age of these patients was 57, 61% were female and 92% had extra-

thoracic metastatic disease. Mean reads per sample was 8.9 million, with a mean coverage per base of 983 unique reads.

The sensitivity of the NGS platform was first studied in 29 of the 48 patients known by tumor genotyping to harbor *EGFR* and *KRAS* driver mutations readily assayed with ddPCR (Fig. 2A). Using the tumor genotyping as the gold standard, ddPCR of plasma had a sensitivity of 86%, while NGS had a sensitivity of 79%, not significantly different (p=0.43). Both methods were more sensitive when more cfDNA was available. The allelic fraction of the mutant allele in cfDNA, calculated as the number of mutant reads over wildtype reads, was closely correlated for plasma NGS and plasma ddPCR (Pearson Correlation = 0.93, p<0.001, Fig. 2B).

Detection of rare mutations and rearrangements in cfDNA was next studied in a blinded fashion in 20 of the 48 patients with NSCLC known to harbor a rare mutation or rearrangement on tumor genotyping (Fig. 2C); these included 19 new cases plus one previously studied above, harboring both a *KRAS* mutation and a *PIK3CA* mutation. Plasma NGS was able to blindly detect 6 out of 8 cases with rearrangements as well as 4 cases with rare targetable *HER2* and *EGFR* mutations. Sensitivity in this cohort was 75% (15/20), similar to the prior analysis, (Fig. 2C). For each of the 6 rearrangements detected, the exact breakpoints and fusion partners could be mapped to the genome (Fig. 3A, Supplementary Fig. 4).

Specificity was studied in these 48 patients, each with tumor genotyping positive for an oncogenic driver mutation in *EGFR*, *KRAS*, *ALK*, *ROS1*, *RET*, *BRAF*, or *HER2*. These oncogenes are established as non-overlapping on tumor genotyping and are therefore ideal gold standards for assessment of false positives.(5, 7) Specificity of the plasma NGS platform was 100% for these seven driver genotypes, with a false positive rate of 0% (95% CI 0-10%).

Detection of resistance mutations was explored in 15 of the 48 patients who had plasma collected after development of acquired resistance to a tyrosine kinase inhibitor (Table 1). Of 12 patients with acquired resistance to erlotinib or afatinib, plasma NGS detected T790M in 8. One of the 12 patients had been refractory to erlotinib and afatinib despite harboring an *EGFR* exon 19 deletion, and tumor NGS had identified high *MET* amplification. Blinded to the tumor genotype, plasma NGS similarly detected *MET* amplification, as evidenced by a significant increase in *MET* copies compared to control (Fig. 3B). No resistance mutations were identified in the remaining 3 *EGFR*-mutant cases. Studying two patients who had developed acquired resistance to crizotinib, plasma NGS identified two point mutations in the *ALK* kinase domain in one case with an ALK rearrangement, and no *ROS1* resistance mutations in the other case with a ROS1 rearrangement. One patient was studied who had previously developed T790M-positive resistance to erlotinib, and subsequently developed acquired resistance to the investigational EGFR kinase inhibitor AZD9291;(20) plasma NGS identified two different DNA mutations encoding for *EGFR* C797S (Fig. 3C), a mutation recently described as a common mediator of acquired resistance to AZD9291.(21). Fourteen of fifteen cases had resistance biopsies available for genotyping. Tumor and plasma results were concordant in 12 (86%), while in 2 cases plasma NGS did not detect an acquired

T790M mutation detected in tumor. Altogether, sensitivity for the 62 known driver and resistance mutations from the 48 cases was 77% (48/62).

Lastly, the plasma NGS assay was explored in two advanced NSCLC patients with high clinical suspicion of possessing a targetable genomic alteration which had been missed on prior tissue genotyping. The initial patient was a 66 year-old female never-smoker who had responded durably to empiric treatment with erlotinib and developed resistance; *EGFR* genotyping of a re-biopsy using a commercial PCR assay had identified T790M without any sensitizing mutation evident. Plasma ddPCR was first performed and detected 1508 copies/ml of an exon 19 deletion. Plasma NGS then confirmed the presence of a novel double-deletion in exon 19 of *EGFR* which would have been missed by many commercial PCR assays because these often detect only common exon 19 deletion variants (Fig. 3D). The second patient was a 28 year-old female never-smoker who had progressed on multiple lines of therapy, for whom previous tumor genotyping (including NGS) had revealed no targetable alterations despite 4 biopsies. Plasma NGS revealed high level *MET* amplification which was subsequently confirmed by fluorescent *in situ* immunohistochemistry (MET:CEP7 ratio >5), and led to the initiation of crizotinib. For each case, turnaround time from blood draw to result reporting in this initial feasibility study was 6 business days.

Conclusion

In this blinded study, a targeted NGS of cfDNA from advanced NSCLC patients was able to accurately detect a broad range of targetable genomic alterations in NSCLC, including point mutations, insertions / deletions, and rearrangements, with no false positives. This report is the first, to our knowledge, to describe the accurate detection of *ALK*, *ROS1*, and *RET* rearrangements using a single plasma assay without prior knowledge of fusion partners. This represents a dramatic advance over PCR-based plasma genotyping assays which are limited to the detection of hotspot mutations in coding regions.(10) This assay was also able to detect both canonical and novel resistance mechanisms, including *MET* amplification and *EGFR* C797S.(22, 23)

Importantly, this approach uses widely available equipment such as standard EDTA-containing vacutainers and a desktop sequencing platform: any accredited molecular pathology lab with a MiSeq could, with the right technical expertise and bioinformatics support, implement this assay to guide the care of advanced NSCLC patients.

While others have also studied plasma NGS of lung cancer, this is the first to describe comprehensive and blinded detection of a broad range of alterations with one clinic-ready assay. Detection of hotspot mutations using plasma NGS was described by Couraud et al using the IonTorrent platform, achieving a sensitivity of 58% and 87% specificity but without the ability to detect rearrangements or amplifications.(11) Newman *et al* recently demonstrated more comprehensive detection of lung cancer mutations in cfDNA and tumor tissue by hybrid capture using biotinylated oligonucleotide probes on a HiSeq;(18) however, this study noted an inefficient capture of fusion rearrangements. Finally, targeted sequencing of cfDNA using PCR amplicons has been successful for detection of SNVs in multiple types of cancer;(15) however, PCR amplicons are not trivial to multiplex and are inherently blind

to gene rearrangements. The NGS platform described here overcomes the weaknesses of each of these prior approaches, allowing multiplexed detection of a broad range of alterations with no false positives using an efficient platform. Furthermore, turnaround time from time of blood draw can be as short as 6 days.

Intuitively, we found that the sensitivity of plasma NGS is improved in specimens with a higher quantity of cfDNA, though in some instances targetable genotypes could be detected in specimens with a relatively low number of GE sequenced indicating high DNA shed from the tumor. In this pilot study, sensitivity of plasma NGS was 77%, comparable to most plasma genotyping assays which have reported sensitivity in the range of 70%.(24), Sensitivity improves with higher DNA concentration, highlighting how important it will be to understand why the amount of total cfDNA in plasma varies across such a wide range, and whether this variance is due to fluctuations in tumor biology or in methods of extraction. For example, we and others have described previously that plasma genotyping assays are more sensitive in lung cancer patients with extra-thoracic metastases.(13, 15, 17, 18) Even with a moderately high sensitivity, the lack of false positives with this assay results in a 100% positive predictive value, meaning that this plasma NGS assay could be used as a screening step before a biopsy sample is taken for genomic analysis: if positive, the results are reliable and can potentially obviate the need for a biopsy, and if negative, a biopsy for further testing may still have value.

Our detection of various resistance mechanisms in patients with acquired resistance to TKIs, including the detection of two different *EGFR* C797S clones in one case, highlights the potential power of plasma NGS for understanding the heterogeneity of the resistant state. In addition, the C797S alleles identified with plasma NGS were clearly in *cis* with T790M (Figure 3), a detail that would be difficult to decipher with a PCR assay and may have treatment implications. (22) It is increasingly appreciated that resistant tumors are made up of clones with diverse biologies that may respond differently to therapy.(25, 26) We recently showed that three molecular subtypes of acquired resistance to the investigational *EGFR* inhibitor AZD9291 are apparent by use of serial plasma ddPCR: while all patients started with T790M plus a sensitizing mutation, some lose T790M at resistance while some gain C797S.(21) However, this analysis required five separate PCR assays: T790M, 19 deletion, L858R, and assays for two C797S variants. Alternatively, one plasma NGS assay can detect these 5 alterations plus detecting any novel resistance mechanisms that emerge. Our data further suggest that quantification of allelic fraction is similar using plasma NGS and ddPCR, suggesting either could be used to serially monitor plasma genotype concentration.

The findings described in this report are achieved by applying a novel bias-corrected capture technology that builds upon standard sequencing approaches to maximize the efficiency of on-target versus off-target and redundant sequencing reads. By reducing PCR artifacts, this assay can accurately detect mutation present in as few as 0.1% of sequencing reads; in contrast, some tumor NGS platforms are unable to accurately call a mutation unless detected in >10% of sequencing reads. Bias-corrected targeted NGS is an approach that can be applied to any sequencing platform to improve on-target coverage and reduce noise. Here, we apply bias-corrected targeted NGS to desktop sequencing on a MiSeq platform in order to develop a plasma assay with the potential to be rapid and clinic-ready, in contrast to more

time intensive assays utilizing HiSeq platforms.(18) Yet this NGS approach could also be applied to clinical sequencing panels or discovery efforts to improve sequencing coverage and reduce artifact, particularly when studying clinical specimens from small biopsies with scant DNA.

In conclusion, we have developed and successfully piloted a plasma NGS assay that, using a novel capture and analysis technique, can detect targetable mutations and rearrangements in plasma from advanced NSCLC patients. This is the first plasma NGS assay to demonstrate blinded, multiplexed detection of such a broad range of actionable alterations with no false positive results. By using a widely available desktop sequencing platform and standard vacutainers with the potential for a rapid turnaround time, this assay has the potential for broad uptake and application. Through reducing the barriers between NSCLC patients and genotyping, we hope that plasma NGS will be able to facilitate delivery of targeted therapies and improve outcomes for patients with advanced NSCLC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

Noninvasive genotyping of cell-free plasma DNA (cfDNA) is a potentially powerful tool for advancing cancer care and translational research, but most established assays are PCR-based and limited to detection of hotspot mutations. Here, we describe the development of a novel rapid targeted next-generation sequencing (NGS) assay for study of cfDNA. Studying 48 cases using a desktop sequencer, this assay was able to detect targetable oncogenic genomic alterations and resistance mechanisms in advanced non-small cell lung cancer without any false positives. The comprehensive coverage afforded by this assay while utilizing a widely available NGS platform has great potential for broad uptake as a tool for noninvasive tumor genotyping.

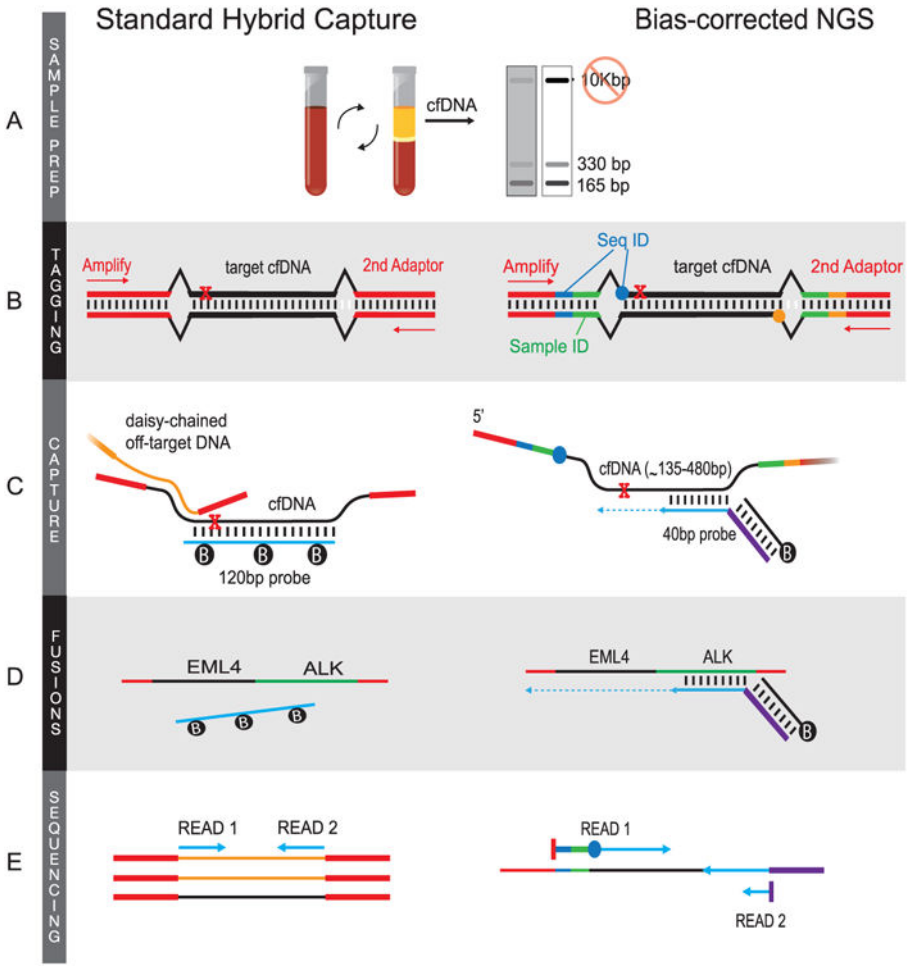

Fig 1. Key differences between standard hybrid capture (left) and bias-corrected NGS (right)

(A) Mono-, di- and trimeric nucleosome cfDNA fragments ranging from 130-480 basepairs are isolated. (B) In standard hybrid capture, cfDNA fragments are end-repaired and ligated with single primers. In contrast, bias-corrected NGS uses multifunctional adaptors that include sequences for single-primer amplification (red), tags for sample identification (green), and sequence identification tags (blue) that, in conjunction with the fragmentation site (blue dot) identify unique sequence clones. (C) In standard hybridization cfDNA fragments are captured with large capture probes (up to 120 bp) that span the genetic region of interest and may result in off-target fragments being isolated (e.g., daisy-chaining off-target DNA). Bias-corrected NGS uses small capture probes (~40 bp) that are designed to be adjacent to the region of interest. Primer extension of fragments copies genomic and adaptor sequences. Lastly, amplification with tailed PCR primers create sequencing ready clones. (D) While both approaches allow sequencing of gene re-arrangements, large capture probes designed to target one gene will inefficiently target fragments containing a large amount of fusion partner gene sequence, resulting in poor sensitivity. In bias-corrected NGS, gene junction and partner gene sequence is replicated during primer extension. E: In standard hybrid capture all pulled-down cfDNA (specific and non-specific) is amplified and sequenced without knowing the exact read or probe which captured the fragment. In bias-corrected NGS, READ_1 identifies the sample ID and the unique sequence identifiers, while

READ_2 identifies the probe that pulled down each clone, facilitating read analysis and probe optimization.

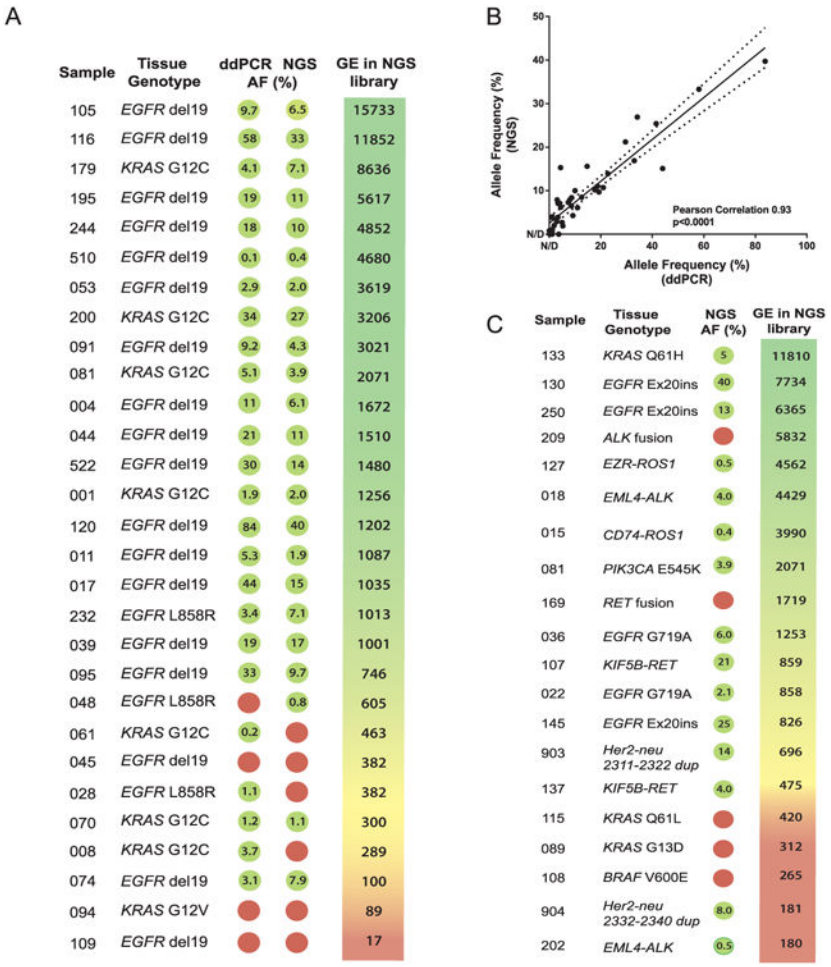

Fig. 2.

Plasma NGS compared to known tumor genotype across a range of genomic equivalents (GE) in the sequencing library. The mutant allele frequency is provided when detected by the plasma genotyping assay (green circle) but not if undetected (red circle). In patients with common *EGFR* and *KRAS* mutations (A) plasma NGS has similar sensitivity to plasma ddPCR. Quantification of allelic frequency with plasma NGS and plasma ddPCR are closely correlated (B). In patients with rare genotypes (C), plasma NGS is able to detect a wide range of genomic alterations. In both groups of patients, the rate of detection by plasma NGS increases as the number of GE increases (A, C).

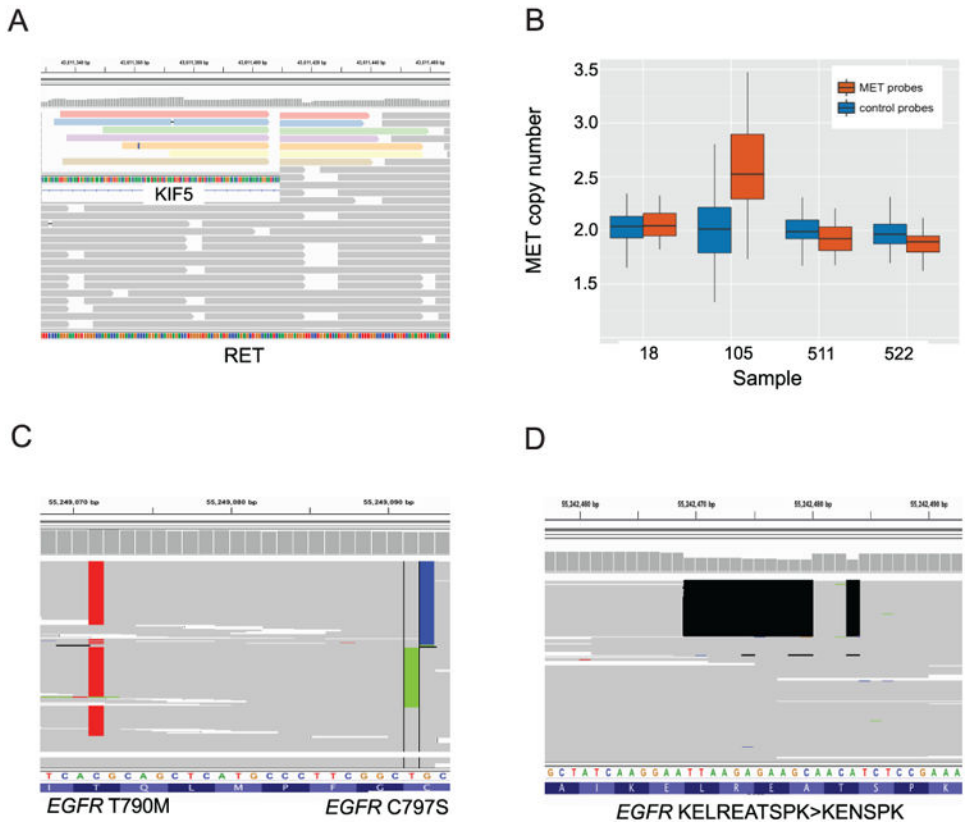

Fig. 3.

Bias-corrected NGS of cfDNA identifies complex genomic alterations. (A) Sequencing of the intronic region of *RET* detects reads extending into KIF5B (inset), predicting a fusion of these two genes. (B) In cfDNA from a case with known *MET* amplification (case 105), *MET* copy number is significantly increased compared to control probes ($p<0.001$), which is not seen in cases without *MET* amplification. (C) Two mutations encoding for *EGFR* C797S are detected in *cis* with *EGFR* T790M after resistance to AZD9291. (D) In a case with acquired T790M despite no apparent *EGFR* sensitizing mutation, plasma NGS detects a novel double-deletion in exon 19 of *EGFR* which would have been missed with many PCR-based genotyping assays.

Table 1

Detection of resistance mechanisms using plasma NGS compared to tissue genotyping of re-biopsy specimens in patients with acquired resistance to kinase inhibitors.

Sample	Baseline genotype	Therapy received	Tissue genotype at resistance	Plasma NGS at resistance	Allele frequency
11	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	1.9% 0.5%
17	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	15.1% 10.0%
39	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	9.7% 2.7%
44	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	10.7% 7.3%
48	*EGFR* L858R	afatinib	*EGFR* L858R *EGFR* T790M	*EGFR* L858R	0.8%
74	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del	7.9%
91	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	4.3% 3.9%
95	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	16.9% 15.6%
22	*EGFR* G719A	erlotinib	*EGFR* G719A	*EGFR* G719A	2.1%
105	*EGFR* exon 19 del	afatinib	*EGFR* exon 19 del *MET* amp	*EGFR* exon 19 del *MET* amp	6.5% NC
522	*EGFR* exon 19 del *EGFR* T790M	AZD9291	*EGFR* exon del 19 *EGFR* T790M *EGFR* C797S	*EGFR* exon del 19 *EGFR* T790M *EGFR* C797S	13.9% 8.5% 7.8%
18	*ALK* rearrangement	crizotinib	(No tissue available)	*EML4-ALK* *ALK* G1128A *ALK* G1156Y	4.0% 2.0% 1.6%
127	*ROS1* rearrangement	crizotinib	*ROS1-EZR*	*ROS1-EZR*	0.5%
120	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	39.7% 8.4%
232	*EGFR* L858R	erlotinib	*EGFR* L858R *EGFR* T790M	*EGFR* L858R *EGFR* T790M	7.1% 6.2%

Original Investigation

Prospective Validation of Rapid Plasma Genotyping for the Detection of *EGFR* and *KRAS* Mutations in Advanced Lung Cancer

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IMPORTANCE Plasma genotyping of cell-free DNA has the potential to allow for rapid noninvasive genotyping while avoiding the inherent shortcomings of tissue genotyping and repeat biopsies.

OBJECTIVE To prospectively validate plasma droplet digital PCR (ddPCR) for the rapid detection of common epidermal growth factor receptor (*EGFR*) and *KRAS* mutations, as well as the *EGFR* T790M acquired resistance mutation.

DESIGN, SETTING, AND PARTICIPANTS Patients with advanced nonsquamous non–small-cell lung cancer (NSCLC) who either (1) had a new diagnosis and were planned for initial therapy or (2) had developed acquired resistance to an EGFR kinase inhibitor and were planned for rebiopsy underwent initial blood sampling and immediate plasma ddPCR for *EGFR* exon 19 del, L858R, T790M, and/or *KRAS* G12X between July 3, 2014, and June 30, 2015, at a National Cancer Institute–designated comprehensive cancer center. All patients underwent biopsy for tissue genotyping, which was used as the reference standard for comparison; rebiopsy was required for patients with acquired resistance to EGFR kinase inhibitors. Test turnaround time (TAT) was measured in business days from blood sampling until test reporting.

MAIN OUTCOMES AND MEASURES Plasma ddPCR assay sensitivity, specificity, and TAT.

RESULTS Of 180 patients with advanced NSCLC (62% female; median [range] age, 62 [37-93] years), 120 cases were newly diagnosed; 60 had acquired resistance. Tumor genotype included 80 *EGFR* exon 19/L858R mutants, 35 *EGFR* T790M, and 25 *KRAS* G12X mutants. Median (range) TAT for plasma ddPCR was 3 (1-7) days. Tissue genotyping median (range) TAT was 12 (1-54) days for patients with newly diagnosed NSCLC and 27 (1-146) days for patients with acquired resistance. Plasma ddPCR exhibited a positive predictive value of 100% (95% CI, 91%-100%) for *EGFR* 19 del, 100% (95% CI, 85%-100%) for L858R, and 100% (95% CI, 79%-100%) for *KRAS*, but lower for T790M at 79% (95% CI, 62%-91%). The sensitivity of plasma ddPCR was 82% (95% CI, 69%-91%) for *EGFR* 19 del, 74% (95% CI, 55%-88%) for L858R, and 77% (95% CI, 60%-90%) for T790M, but lower for *KRAS* at 64% (95% CI, 43%-82%). Sensitivity for *EGFR* or *KRAS* was higher in patients with multiple metastatic sites and those with hepatic or bone metastases, specifically.

CONCLUSIONS AND RELEVANCE Plasma ddPCR detected *EGFR* and *KRAS* mutations rapidly with the high specificity needed to select therapy and avoid repeat biopsies. This assay may also detect *EGFR* T790M missed by tissue genotyping due to tumor heterogeneity in resistant disease.

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Editorial

Supplemental content at jamaoncology.com

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Plasma genotyping uses tumor-derived cell-free DNA (cfDNA) to allow for rapid noninvasive genotyping of tumors. This technology is currently poised to transition into a treatment decision-making tool in multiple cancer types. It is particularly relevant to the treatment of advanced non–small-cell lung cancer (NSCLC), in which therapy hinges on rapid and accurate detection of targetable epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), and *ROS1* alterations.[1-6] Plasma genotyping is capable of circumventing many limitations of standard tissue genotyping including slow turnaround time (TAT), limited tissue for testing, and the potential for failed biopsies. It may be particularly useful in directing the rapid use of new targeted therapies for acquired resistance in advanced *EGFR*-mutant NSCLC, where the need for a repeat biopsy to test for resistance mechanisms has amplified the inherent limitations of traditional genotyping.[7,8]

The need to carefully validate the test characteristics of each of the myriad individual plasma genotyping assays before use in clinical decision making is paramount. We have previously reported the development of a quantitative droplet digital polymerase chain reaction (ddPCR)-based assay for the detection of *EGFR* kinase mutations and *KRAS* codon 12 mutations in plasma.[9] The detection of these mutations has the potential to guide treatment by either facilitating targeted therapy with an EGFR tyrosine kinase inhibitor (TKI) or ruling out the presence of other potentially targetable alterations in the case of *KRAS*.[5] Alternative platforms including Cobas, peptide nucleic acid–mediated PCR, multiplexed next-generation sequencing (NGS), high-performance liquid chromatography, and Scorpion–amplified refractory mutation system have also been examined in retrospective analyses of patient samples.[10-22] The test characteristics of these assays have been variable and may be attributable to differences in testing platforms, as well as the retrospective nature of these studies, their smaller size, and the timing of blood collection with respect to disease progression and therapy initiation. The absence of reliable prospective data on the use of specific plasma genotyping assays in advanced NSCLC has left key aspects of its utility largely undefined and slowed its uptake as a tool for clinical care in patients with both newly diagnosed NSCLC and EGFR acquired resistance.

To our knowledge, we have conducted the first prospective study of the use of ddPCR-based plasma genotyping for the detection of *EGFR* and *KRAS* mutations. This study was performed in the 2 settings where we anticipate clinical adoption of this assay: (1) patients with newly diagnosed advanced NSCLC and (2) those with acquired resistance to EGFR kinase inhibitors. The primary aim of this study was to prospectively evaluate the feasibility and accuracy of this assay for the detection of *EGFR/KRAS* mutations in patients with newly diagnosed NSCLC and *EGFR* T790M in patients with acquired resistance in a clinical setting. Additional end points included test TAT and the effect of sample treatment conditions on test accuracy.

Key Points

Question What is the sensitivity, specificity, turnaround time, and robustness of droplet digital polymerase chain reaction (ddPCR)-based plasma genotyping for the rapid detection of targetable genomic alterations in patients with advanced non–small-cell lung cancer (NSCLC)?

Findings In this study of 180 patients with advanced NSCLC (120 newly diagnosed, 60 with acquired resistance to epidermal growth factor receptor [EGFR] kinase inhibitors), plasma genotyping exhibited perfect specificity (100%) and acceptable sensitivity (69%-80%) for the detection of *EGFR*-sensitizing mutations with rapid turnaround time (3 business days). Specificity was lower for *EGFR* T790M (63%), presumably secondary to tumor heterogeneity and false-negative tissue genotyping.

Meaning The use of ddPCR-based plasma genotyping can detect *EGFR* mutations with the rigor necessary to direct clinical care. This assay may obviate repeated biopsies in patients with positive plasma genotyping results.

Methods

Trial Design

Patients with advanced NSCLC were prospectively enrolled onto an institutional review board–approved plasma genotyping study protocol (NCT02279004). Patients were eligible for the study if they had biopsy-proven advanced or recurrent nonsquamous NSCLC and were either treatment naive (cohort 1) or had acquired resistance to an EGFR TKI (cohort 2). All patients must have been planned to begin new systemic therapy and have either tissue available for standard genotyping or a planned repeat biopsy. All patients had radiographic evidence of disease, were 18 years of age or older, and signed written informed consent before any study-related procedure. Participant-defined race was recorded given known associations between race and the frequency of *EGFR*-mutant tumors.

All patients underwent an initial paired blood collection after study enrollment. These 2 tubes of blood subsequently underwent plasma isolation, cfDNA extraction, and ddPCR-based genotyping. One tube of blood was processed and analyzed immediately in accordance with standard operating procedures, and the second was subjected to preplanned variations in specimen handling designed to simulate real-world testing conditions including (1) standard ethylenediaminetetraacetic acid (EDTA) tube shipped overnight on ice and (2) Streck tube shipped at room temperature. Comparison between paired samples was made on the basis of sample quality, total DNA as determined by PicoGreen assay, and quantitative ddPCR result. Differences between paired tubes were analyzed by paired *t* test. If more than 2 weeks elapsed before initiation of planned systemic therapy, the blood sampling was repeated. The TAT for plasma genotyping was measured in business days from the date of blood sampling until reporting of results to the study investigator.

Patient samples from cohort 1 underwent ddPCR-based plasma genotyping for *EGFR* exon 19 del, L858R, and *KRAS* codon 12 mutations. Cohort 2 samples underwent testing for *EGFR* exon 19 del, L858R, and T790M. Plasma genotyping results were compared with tissue genotyping results from initial biopsy (cohort 1) or rebiopsy at acquired resistance (cohort 2) as the reference standard.

Patients from both cohorts who had a mutation detected by ddPCR-based genotyping subsequently underwent 2 follow-up blood draws at 1 to 2 weeks and 4 to 6 weeks after beginning systemic therapy. These samples underwent serial quantitative genotyping by ddPCR for the detected mutation.

Plasma Genotyping

Venous blood samples were collected in EDTA tubes and underwent centrifugation within 1 hour of sample collection and plasma preparation as previously described.[9] Immediate extraction of cfDNA was then performed using the QIAmp circulating nucleic acid kit (QIagen) according to the manufacturer's protocol. DNA was eluted in 100 μL of AVE buffer and stored at −80°C until genotyping was performed. Genotyping of cfDNA was performed by ddPCR (BioRad) and primer/probes were custom ordered from Life Technologies. The development of this assay has been previously described.[9] Briefly, cfDNA is emulsified into approximately 20 000 droplets, mixed with appropriate primer/probe mixes, and then undergoes PCR to end point. Droplets are then read in a flow cytometer and fluorescence signal quantified to determine the number of copies of mutant allele per milliliter (eMethods in the Supplement). To simulate standard clinical practice, the assay was performed twice weekly (Monday and Thursday). Laboratory personnel performing plasma ddPCR were blinded to tissue genotyping results.

Tissue Genotyping

Clinical tumor genotyping was performed for all patients on initial biopsy material (cohort 1) or rebiopsy material following development of acquired resistance (cohort 2). Turnaround time for tissue genotyping was measured from the date of the initial genotyping order until the reporting of the final genotyping result. In cases in which a repeat biopsy was required to successfully complete tissue genotyping, the time required to perform the repeat biopsy was included in the TAT measurement.

Statistical Analysis

From a total of 120 patients with NSCLC studied in cohort 1, we estimated that 24 and 30 would have *EGFR* and *KRAS* mutations, respectively, based on prior data at our institution. Concordance between tumor and plasma genotyping results had at least 80% power to detect a κ statistic of 0.85 (compared with a null of 0.60) while controlling for a 1-sided type 1 error rate of .15.

For the 60 patients with acquired resistance who were planned for cohort 2, we estimated that half would harbor T790M detected in their resistance biopsy. An expanded target of 80 patients was originally planned but was revised to 60 patients as a result of feasibility concerns. Concordance between tumor and plasma genotyping results for T790M had 88% power to detect a κ coefficient of 0.85 (compared with a null of 0.60) while controlling for a 1-sided type 1 error rate of .05. Categorical variables were compared using the Fisher exact test, and continuous variables were compared using the Wilcoxon rank sum test or Kruskal-Wallis test. No adjustments have been made for multiple comparisons.

Results

Patients

A total of 180 patients with advanced NSCLC were enrolled in the study with either newly diagnosed disease (n = 120) or acquired resistance to an EGFR TKI (n = 60). Most patients had adenocarcinoma histologic subtype (169 [94%]), and only a few had either NSCLC not otherwise specified (5 [3%]) or adenosquamous histologic subtype (6 [3%]) (**Table 1**). Patients were predominantly female (112 [62%]) and primarily white (152 [84%]) or Asian (20 [11%]). Patients who did not complete their initial blood sampling or any tissue genotyping were excluded from the analysis (eFigure 1 in the Supplement). An additional 28 patients did not have sufficient tissue available for *KRAS* G12X testing after completion of initial *EGFR* testing and were excluded from the *KRAS* G12X analysis.

The confirmed tissue genotypes of the 115 eligible patients with newly diagnosed NSCLC included 14 *EGFR* exon 19 del, 13 *EGFR* L858R, 26 *KRAS* G12X, and 62 *EGFR*/*KRAS* wild type (Table 1). The 54 eligible patients with acquired resistance possessed a range of *EGFR* sensitizing mutations (37 *EGFR* exon 19 del, 18 *EGFR* L858R, 5 rare) and 35 (58%) of these patients were *EGFR* T790M positive according to tissue genotyping performed on rebiopsy specimens.

Turnaround Time and Repeat Biopsy

Plasma genotyping was completed successfully in all patients. The median (range) TAT from blood collection to report delivery was 3 (1-7) business days in patients with newly diagnosed NSCLC and 2 (1-4) business days in patients with acquired resistance. In comparison, the median (range) TAT for tissue genotyping in patients with newly diagnosed NSCLC was significantly longer at 12 (1-54) business days ($P < .001$). The median (range) TAT for tissue genotyping was similarly longer in patients with acquired resistance to EGFR kinase inhibitors at 27 (1-146) business days. A repeat biopsy was required in 22 (19%) patients with newly diagnosed NSCLC to obtain sufficient tissue to complete genotyping. Similarly, 12 (21%) patients with acquired resistance required multiple biopsies to obtain sufficient tissue for *EGFR* T790M genotyping. Turnaround time measurements included the time required to obtain an additional biopsy if necessary due to failure of 1 or more biopsy attempts.

Assay Characteristics

The accuracy of the *EGFR* exon 19 del, L858R, and *KRAS* G12X assays was studied first in patients with newly diagnosed NSCLC (n = 115) (eFigure 1 in the Supplement). Plasma ddPCR exhibited high specificity for the detection of *EGFR* exon 19 del (100% [101 of 101]), L858R (100% [102 of 102]), and *KRAS* G12X (100% [62 of 62]). Positive predictive value was similarly high for all assays at 100% (**Table 2**). Assay sensitivity was more modest for *EGFR* exon 19 del (86% [12 of 14]), L858R (69% [9 of 13]), and *KRAS* G12X (64% [16 of 25]) (Table 2). Concordance was 0.91 ($P = .01$) for *EGFR* exon 19 del, 0.80 ($P = .08$) for L858R, and 0.72 ($P = .13$) for *KRAS* G12X. Assay sensitivity among patients with newly diag-

Table 1. Patients Characteristics

Characteristics	Total (N = 180)[a]	Cohort 1, Newly Diagnosed (n = 120)	Cohort 2, Acquired Resistance (n = 60)
Sex, No. (%)			
Male	68 (38)	50 (42)	18 (30)
Female	112 (62)	70 (58)	42 (70)
Age, median (range), y	62 (37-93)	64 (37-93)	58 (38-81)
Race, No. (%)			
White	152 (84)	109 (91)	43 (72)
Asian	20 (11)	7 (6)	13 (22)
Black	5 (3)	2 (2)	3 (5)
Hispanic	3 (2)	2 (2)	1 (1)
Stage at diagnosis, No. (%)			
Recurrent	5 (3)	5 (4)	0
IIIB	3 (2)	3 (3)	0
IV	172 (95)	112 (93)	60 (100)
No. of metastatic sites, No. (%)			
1	72 (40)	55 (46)	17 (28)
2	62 (34)	41 (34)	21 (35)
3	23 (13)	15 (12)	8 (13)
>4	23 (13)	9 (8)	14 (23)
Site of biopsy used for genotyping, No. (%)			
Lung	76 (42)	47 (39)	29 (53)
Pleural fluid	15 (8)	12 (10)	3 (5)
Liver	13 (7)	7 (6)	6 (10)
Lymph node	36 (20)	28 (23)	8 (14)
Other	36 (20)	26 (22)	10 (18)
Tissue genotype, No. (%)[b]			
EGFR exon 19 deletion	50 (28)	14 (12)	36 (62)
EGFR L858R	31 (18)	13 (11)	18 (30)
Rare *EGFR* mutation	5 (3)	0	5 (8)
EGFR T790M	35 (19)	0	35 (58)[c]
KRAS G12X	26 (14)	26 (22)	0
EGFR/*KRAS* wild type	64 (36)	64 (53)	0
Failed genotyping	3 (1)	3 (3)	5 (8)[d]
Tissue genotyping method, No. (%)			
Sanger sequencing	6 (3)	6 (5)	0
PCR	72 (40)	29 (25)	43 (78)
Targeted NGS	24 (13)	12 (10)	12 (22)
PCR and NGS	70 (39)	70 (60)	0
Additional biopsy required	34 (19)	22 (19)	12 (21)
Histologic subtype			
Adenocarcinoma	169 (94)	112 (93)	57 (95)
Adenosquamous	6 (3)	3 (3)	3 (5)
NSCLC not otherwise specified	5 (3)	5 (4)	0

Abbreviations: NGS, next-generation sequencing; NSCLC, non–small-cell lung cancer; PCR, polymerase chain reaction.

[a] Thirty patients included in this study were also included in previously reported validation of an alternative plasma NGS assay for cell-free DNA genotyping.[23]

[b] Values may not total 100% because T790M mutation may occur concurrently with other *EGFR* mutations.

[c] Percentage of *EGFR*-mutant patients in this cohort with T790M-positive disease.

[d] Percentage of *EGFR*-mutant patients in this cohort who did not complete biopsy for T790M testing.

nosed disease and acquired resistance was similar for the detection of *EGFR* exon 19 del (82% [41 of 50]) and L858R (74% [23 of 31]) (Table 2). A single false-positive result was initially reported for *EGFR* exon 19 del testing (132 copies/mL), which occurred in a young never-smoker with a scant tumor biopsy sample that was negative for any *EGFR* mutations. A second biopsy was then performed, and subsequent tumor genotyping confirmed an *EGFR* exon 19 del mutation.

The accuracy of the *EGFR* T790M assay was studied in patients with acquired resistance to *EGFR* TKI. The detection of this resistance mutation by plasma ddPCR exhibited a lower specificity (63% [12 of 19]) and positive predictive value (79% [27 of 34]) than was seen for *EGFR* sensitizing mutations when compared with tumor genotyping of the resistance biopsy; thus, concordance was also lower for the detection of *EGFR* T790M (κ statistic, 0.4; $P = .10$). The sensitivity of this assay

Table 2. Plasma Droplet Digital Polymerase Chain Reaction Assay Sensitivity, Specificity, and Positive Predictive Value

Assay	Sensitivity Analysis			Specificity Analysis			Positive Predictive Value, % (95% CI)
	Sensitivity, % (95% CI)	No. True Positive[a]	No. False Negative[b]	Specificity, % (95% CI)	No. True Negative[c]	No. False Positive[d]	
***EGFR* exon 19 del**							
Newly diagnosed	86 (57-98)	12	2	100 (96-100)	101	0	100 (74-100)
Acquired resistance	81 (64-92)	29	7	100 (85-100)	23	0	100 (88-100)
Overall	82 (69-91)	41	9	100 (97-100)	124	0	100 (91-100)
***EGFR* L858R**							
Newly diagnosed	69 (39-91)	9	4	100 (96-100)	102	0	100 (66-100)
Acquired resistance	78 (52-94)	14	4	100 (91-100)	41	0	100 (77-100)
Overall	74 (55-88)	23	8	100 (97-100)	143	0	100 (85-100)
***EGFR* T790M**							
Acquired resistance	77 (60-90)	27	8	63 (38-84)	12	7	79 (62-91)
***KRAS* G12X**							
Newly diagnosed	64 (43-82)	16	9	100 (94-100)	62	0	100 (79-100)

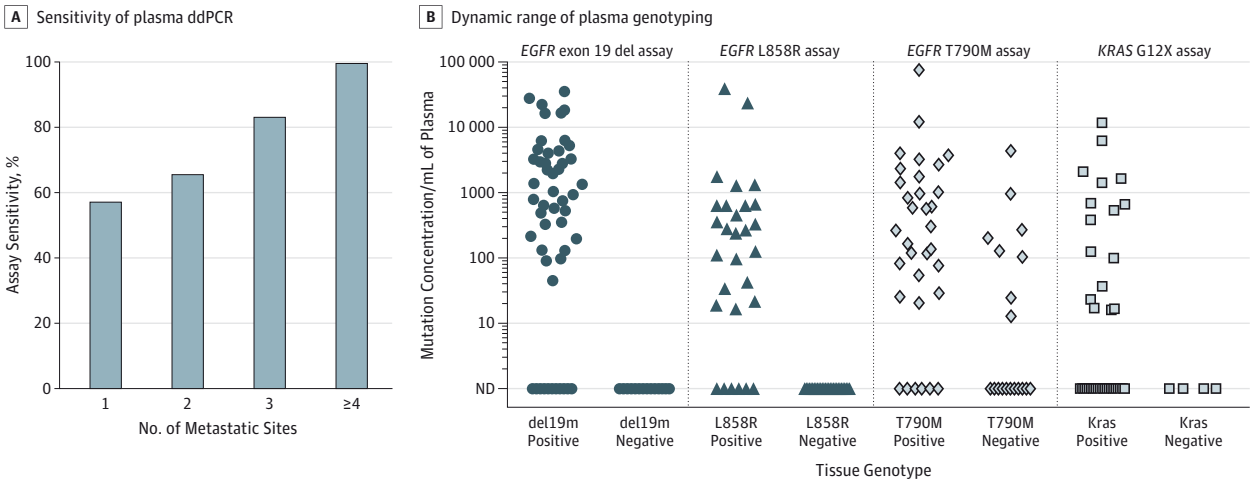
[a] True positive indicates positive test result in both tissue and plasma.

[b] False negative indicates positive test result in tissue and negative result in plasma.

[c] True negative indicates negative test result in both tissue and plasma.

[d] False positive indicates negative test result in tissue and positive result in plasma.

Figure 1. Sensitivity and Dynamic Range of Plasma Droplet Digital Polymerase Chain Reaction (ddPCR) for the Detection of *EGFR* and *KRAS* Mutations



A, The sensitivity of plasma ddPCR for the detection of *EGFR* and *KRAS* mutations increases directly with the number of metastatic sites present in a given patient ($P < .001$). B, Dynamic range of plasma genotyping using a validated ddPCR-based assay. Wide dynamic range and the absence of false-positive test results are noted for the detection of *KRAS* G12X and *EGFR* sensitizing mutations. A small number of false-positive results are seen with the *EGFR* T790M assay—potentially secondary to tumor heterogeneity with respect to acquired resistance mechanisms (n = 174). Each symbol represents 1 patient with the specific mutation listed on the x-axis. CT indicates computed tomography; ND, not detectable.

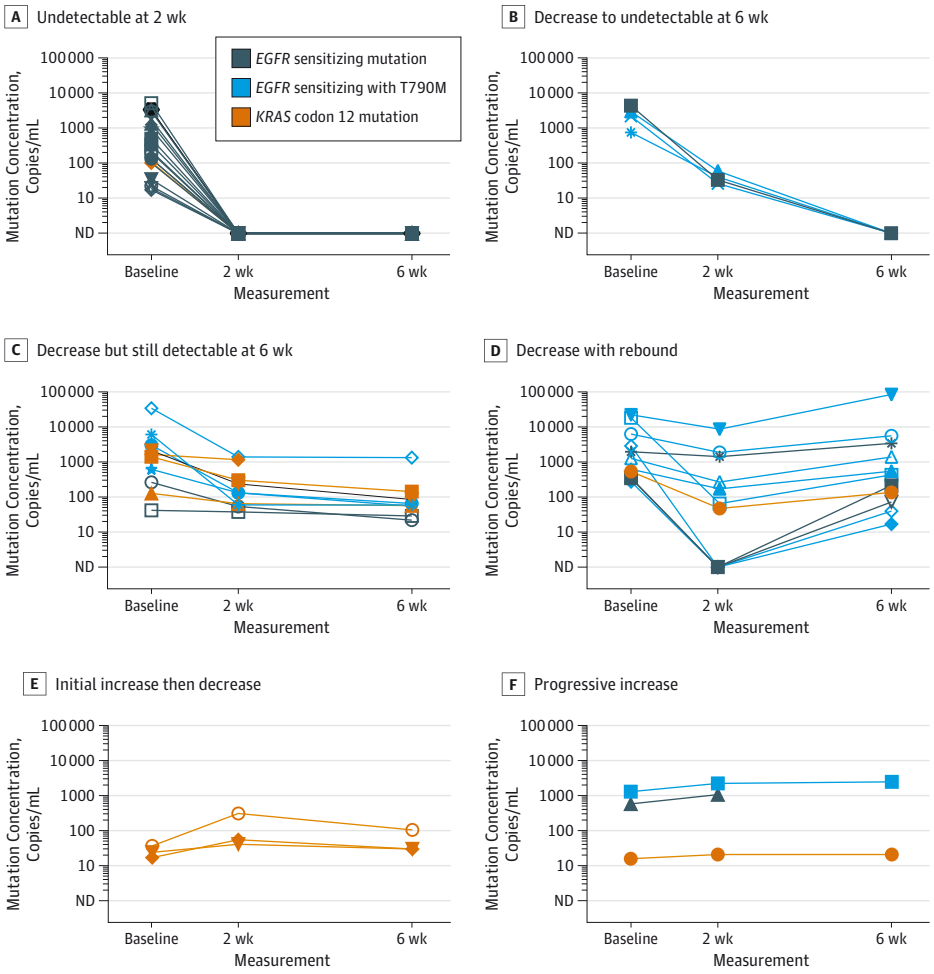
was similar to that observed for *EGFR* sensitizing mutations (77% [27 of 35]) (Table 2). The test characteristics for the detection of *EGFR* sensitizing mutations were similar in patients with acquired resistance compared with patients with newly diagnosed NSCLC (Table 2).

Predictors of Test Sensitivity and Dynamic Range

Patient and disease characteristics potentially associated with increased test sensitivity were examined using a composite test sensitivity variable combining both *EGFR* and *KRAS* assay sensitivity results. Of the variables listed in Table 1, a significant association was demonstrated between test sensitivity and the presence of hepatic metastases ($P = .001$), bone metastases ($P = .007$), and increasing number of metastatic sites ($P = .001$) (**Figure 1**).

The relationship between detected mutant *EGFR* or *KRAS* cfDNA copy number and clinical characteristics was next examined as a marker of tumor cfDNA shed. Given the wide dynamic range noted with this assay (Figure 1), significant associations between clinical characteristics and $\log_{10}$-transformed mutant cfDNA copy number in patients with detected mutant cfDNA were sought. Only increasing number of metastatic sites was associated with a higher mutant cfDNA copy number ($P = .03$).

Figure 2. Distinct Patterns of Plasma Droplet Digital Polymerase Chain Reaction (ddPCR) Plasma Response in Patients Undergoing Serial Plasma Genotyping at 2 and 6 Weeks After Treatment



Six patterns of changes in detectable mutation by plasma ddPCR were observed: A, Mutant cell-free DNA (cfDNA) became undetectable at 2 weeks. B, Mutant cfDNA decreased and then became undetectable at 6 weeks. C, Mutant cfDNA decreased progressively but remained detectable at 6 weeks. D, Mutant cfDNA decreased at 2 weeks and then rebounded at 6 weeks. E, Mutant cfDNA increased initially and then decreased at 6 weeks. F, Mutant cfDNA progressively increased. A and B, Patients with complete resolution of mutant cfDNA exhibited a treatment discontinuation rate of 0% (0 of 23) and 4% (1 of 23) at initial and second restaging computed tomographic (CT) scans. C-F, Alternatively, patients without complete resolution had a treatment discontinuation rate of 33% (9 of 27) at initial reimaging and 56% (15 of 27) at second reimaging assessment. Patient genotypes included *EGFR* sensitizing alone (negative), *EGFR* sensitizing in the presence of T790M (negative), and *KRAS* G12X (negative). ND indicates not detectable.

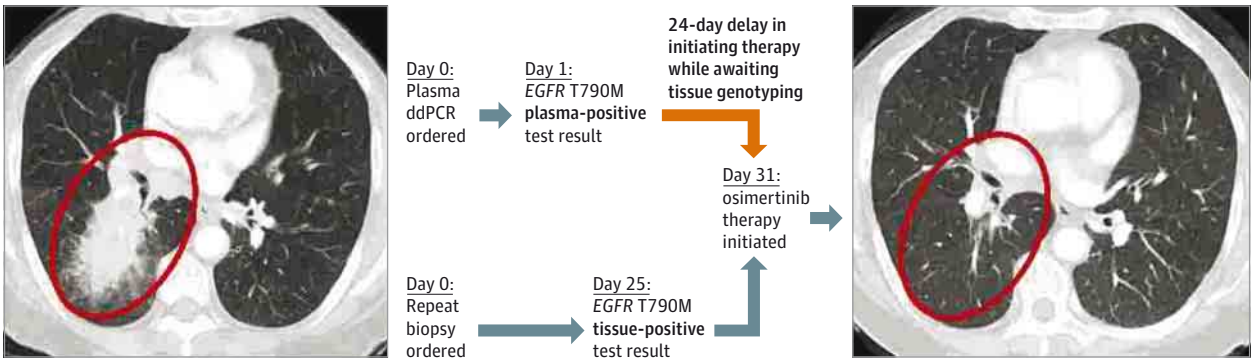
Paired Analysis

Multiple real-world sample treatment conditions were tested using paired samples drawn from the same patient at the same point in time. The use of an EDTA tube that was shipped on ice overnight before processing revealed identical qualitative assay results and not significantly different total DNA (P = .38) and mutant allele copy number (P = .26) compared with immediate processing (n = 25 patients). Similarly, use of a Streck DNA preservation tube shipped at room temperature overnight yielded identical qualitative assay results and there was no significant difference in total DNA (P = .25) or mutant allele copy number (P = .32) compared with standard processing (n = 20 patients) (eFigure 2 in the Supplement).

Exploratory Patterns of Mutant cfDNA Changes in Response to Systemic Therapy

Patients with a detectable mutation by means of plasma ddPCR underwent serial blood sampling on treatment. A total of 50 patients completed at least 1 follow-up blood sampling on treatment. Serial quantitative plasma ddPCR among these patients revealed clear changes in the level of detectable mutant allele frequency during treatment (**Figure 2**). Changes in detectable mutation by plasma ddPCR fell into several recurrent, descriptive patterns including complete resolution of detectable mutant cfDNA either at initial repeated blood sampling (Figure 2A) or subsequently (Figure 2B), residual detectable mutant cfDNA following initial decrease (Figure 2C), initial decrease followed by increase (Figure 2D), or initial increase that was either transient (Figure 2E) or maintained (Figure 2F). Patients with complete resolution of mutant cfDNA at either 2 or 6 weeks exhibited a treatment discontinuation rate of 0% (0 of 23) at initial and 4% (1 of 23) at second reimaging assessment. Patients without complete resolution exhibited a treatment discontinuation rate of 33% (9 of 27) at initial reimaging and 56% (15 of 27) at second reimaging assessment. Treatment discontinuation decisions were made by treating clinicians who were blinded to serial plasma genotyping results. Objective data on overall survival and progression-free survival are presently immature. These patterns of change in plasma response are exploratory at present but provide a potential framework for future analysis of the correlation between changes in detectable mutant cfDNA and response to therapy.

Figure 3. A Woman in Her 80s With Metastatic *EGFR* Mutant Non–Small-Cell Lung Cancer With Acquired Resistance to Erlotinib



Symptomatic progression of pulmonary and bone metastases were noted (primary lung lesion labeled). Empirical single-agent chemotherapy or best supportive care alone were considered given the patient's age and comorbidities. However, plasma droplet digital polymerase chain reaction (ddPCR) was performed and the result returned the next day, revealing 806 copies/mL of *EGFR* T790M. The patient underwent rebiopsy, which confirmed *EGFR* T790M, and the patient was able to start therapy with osimertinib—a novel third-generation EGFR kinase inhibitor—with excellent clinical and radiographic response. Importantly, the plasma ddPCR T790M result was returned 24 days before the results of the repeated tissue biopsy were available.

Discussion

In this prospective study, we demonstrate the highly specific and rapid nature of plasma genotyping. No false-positive test results were seen for driver mutations in *EGFR* or *KRAS*, and TAT from when the specimen was obtained to result was a matter of days. This assay exhibited 100% positive predictive value for the detection of these mutations. Sensitivity was more modest and was directly correlated with both number of metastatic sites and the presence of liver or bone metastases. This newly demonstrated relationship is likely related to increased cfDNA shed in the setting of more extensive disease where tumor cfDNA shed is the chief driver of assay sensitivity and determines its upper limit. The characteristics of plasma ddPCR prospectively demonstrated in this study were similar or improved compared with previous retrospective reports of other cfDNA genotyping assays.[10-13,15,16,24,25] These retrospective studies are smaller, frequently examined a mix of tumor types and/or stages, and lack the careful prospective design needed to demonstrate the readiness of this technology to transition to a tool for selecting therapy. Studies that use retrospective samples from clinical trials that enrolled only *EGFR*-mutant patients are further limited by an inability to both blind laboratory investigators to tissue genotype and to generalize their assay test characteristics to a genetically heterogeneous real-world patient population.[11] These differences and the multiple platforms examined previously have led to variable test characteristics and uncertainty regarding the clinical application of these technologies. This study is the first to prospectively demonstrate the ability of a ddPCR-based plasma genotyping assay to rapidly and accurately detect *EGFR* and *KRAS* mutations in a real-world clinical setting with the rigor necessary to support the assertion that use of this assay is capable of directing clinical care.

Even with a diagnostic sensitivity of less than 100%, such a rapid assay with 100% positive predictive value carries the potential for immense clinical utility. The 2- to 3-day TAT contrasts starkly with the 27-day TAT for tumor genotyping seen in patients needing a new tumor biopsy. This long TAT is due largely to the practical reality that many patients with newly diagnosed NSCLC require a repeat biopsy to obtain tissue for genotyping, as do all patients with acquired resistance. Consider the case of 1 study participant, an octogenarian with metastatic NSCLC who had developed acquired resistance to erlotinib with painful bone metastases (**Figure 3**). Due to the patient's age and comorbidities, significant concerns existed about the risks of a biopsy and further systemic therapy. A plasma sample was obtained, and within 24 hours ddPCR demonstrated 806 copies/mL of *EGFR* T790M. A confirmatory lung biopsy was performed, which confirmed *EGFR* T790M. Treatment with a third-generation EGFR kinase inhibitor, osimertinib mesylate, was subsequently initiated and the patient had a partial response to therapy that was maintained for more than 1 year. The potential of this technology to obviate repeated biopsy in both patients with newly diagnosed NSCLC with insufficient tissue, as well as patients with acquired resistance, is considerable.

A key limitation of plasma ddPCR is that although this method is adept at rapidly detecting specific targetable mutations, it cannot easily detect copy number alterations and rearrangements. The ddPCR panel assessed in this study thus cannot currently detect targetable alterations in either *ALK* or *ROS1*. This limitation may potentially be addressed by using targeted NGS of cfDNA for broad, multiplexed detection of complex genomic alterations including *ALK* and *ROS1* rearrangements, although this method is potentially slower than ddPCR-based methods and has been less thoroughly evaluated.[23] The potential exists to use these technologies in tandem in advanced NSCLC to facilitate rapid initiation of therapy. Tissue

genotyping and repeated biopsy would be specifically used to direct therapy in cases in which plasma genotyping was uninformative due to limitations of assay sensitivity. This approach would be particularly useful in cases of *EGFR* acquired resistance in which a repeated biopsy for T790M testing could be avoided entirely in many patients. Beyond detecting targetable alterations in order to drive therapy, the identification of nontargetable oncogenic drivers such as *KRAS* mutations that preclude the presence of other targetable alterations may guide a clinician to rapidly initiate alternative therapies such as chemotherapy or immunotherapy.[5] The finding that assay sensitivity is highest in patients with more extensive metastatic disease suggests that those patients most in need of rapid treatment initiation would also be least likely to have false-negative results.

One surprising result of our study was evidence of recurrent false-positive results for *EGFR* T790M in patients with acquired resistance, despite no false-positive test results for other mutations studied. The sensitivity of the *EGFR* T790M assay was comparable to that of the *EGFR* sensitizing mutation assays and similarly related to both disease burden and the presence of liver or bone metastases, which are likely predictive of increased tumor cfDNA shed. We hypothesize that the lower assay specificity is due to the genomic heterogeneity whereby the T790M status of the biopsied site is not representative of all metastatic sites in a patient, a phenomenon supported by mounting evidence in the acquired resistance setting.[26,27] This is consistent with the finding that a minority of patients with apparently *EGFR* T790M tissue-negative disease respond to therapy with third-generation EGFR kinase inhibitors.[7,8,28] These observations raise questions regarding the fallibility of tissue-based genotyping as the reference standard for T790M status. The use of plasma genotyping to detect *EGFR* T790M thus has great potential to identify patients who would benefit from newly approved third-generation EGFR kinase inhibitors but would be unable to access them based on falsely negative tissue genotyping results. Indeed, plasma genotyping may allow more reliable assessment of both T790M status as well as the mechanisms of resistance across all sites of a heterogeneous cancer as opposed to a tissue biopsy and is likely to be an essential tool for future trials targeting drug resistance. The potential to avoid a repeat biopsy entirely in patients in whom plasma ddPCR detects T790M further strengthens the utility of this technology, although a repeat biopsy would still be needed in patients with uninformative plasma ddPCR due to limitations with respect to assay sensitivity.

This study also examined the potential of the quantitative nature of ddPCR-based plasma genotyping to allow for the early prediction of treatment response. Distinct patterns of change in mutant allele copy number were observed as early as 2 weeks after treatment and were similar to those reported in other tumor types.[19,20] We hypothesize that these distinct patterns of change in this study will correlate with specific patterns of radiographic response and emergence of acquired resistance and plan to report these data once mature. The observed differences in treatment discontinuation rates observed in this study comparing patients with complete resolution of detectable mutant cfDNA with those with incomplete resolution support this hypothesis. The use of this technology to monitor disease status in real time has potential utility for both routine clinical care, as well as use as an integrated biomarker in early-phase clinical trials.[10]

Conclusions

Droplet digital polymerase chain reaction–based plasma genotyping is a technology that is ready to be used for clinical decision making in patients with advanced NSCLC. This assay is capable of rapidly detecting *EGFR* and *KRAS* mutations with minimal false-positive test results and with the robustness needed for real-world testing. It has great utility for the detection of actionable genomic alterations in patients who are unable to a undergo repeat biopsy and may even detect mutations missed by standard tissue genotyping due to tissue heterogeneity. As third-generation *EGFR* T790M inhibitors come into clinical use, the need for rebiopsy and potential role of plasma genotyping will expand dramatically. Furthermore, the potential combination of rapid ddPCR-based plasma genotyping assays with plasma NGS assays for more comprehensive noninvasive genotyping may represent a new paradigm for clinical genotyping.

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Study concept and design: Sacher, Paweletz, Dahlberg, Oxnard.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Sacher, Paweletz, Dahlberg, Oxnard.
Critical revision of the manuscript for important intellectual content: Sacher, Paweletz, Alden, O'Connell, Feeney, Mach, Jänne, Oxnard.
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