Discrimination of Germline \textit{EGFR} T790M Mutations in Plasma Cell-Free DNA Allows Study of Prevalence Across 31,414 Cancer Patients

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Abstract

Purpose: Plasma cell-free DNA (cfDNA) analysis is increasingly used clinically for cancer genotyping, but may lead to incidental identification of germline-risk alleles. We studied \textit{EGFR} T790M mutations in non–small cell lung cancer (NSCLC) toward the aim of discriminating germline and cancer-derived variants within cfDNA.

Experimental Design: Patients with \textit{EGFR}-mutant NSCLC, some with known germline \textit{EGFR} T790M, underwent plasma genotyping. Separately, deidentified genomic data and buffy coat specimens from a clinical plasma next-generation sequencing (NGS) laboratory were reviewed and tested.

Results: In patients with germline T790M mutations, the T790M allelic fraction (AF) in cfDNA approximates 50%, higher than that of \textit{EGFR} driver mutations. Review of plasma NGS results reveals three groups of variants: a low-AF tumor group, a heterozygous group (~50% AF), and a homozygous group (~100% AF).

Conclusion: With appropriate bioinformatics, plasma genotyping can accurately predict the presence of incidentally detected germline risk alleles. This finding in patients indicates a need for genetic counseling and confirmatory germline testing.

Introduction

Genomic analysis of cancer specimens has been widely adopted as a tool for guiding precision cancer care. Many academic and commercial labs now offer next-generation sequencing (NGS) panels (1, 2) to identify targetable somatic mutations. One limitation of tumor tissue NGS is the frequent inability to clearly distinguish whether detected variants represent somatic events, potentially driving tumor growth, or underlying germline events, potentially representing inherited risk alleles or benign inherited polymorphisms (3). Indeed, some have advocated for routine paired analysis of tumor DNA and germline DNA to better distinguish potentially targetable somatic alterations (3). However, sequencing of germline DNA requires collection of an additional germline biospecimen, increases cost, and may require additional patient consent due to ethical considerations (4).

One rapidly emerging alternative to tumor tissue genotyping is genomic analysis of plasma cell-free DNA (cfDNA), which can noninvasively identify targetable somatic genomic variants. Because there is variability from patient to patient in regards to how much tumor DNA is shed into the plasma, plasma cfDNA represents a dynamic mixture of germline cfDNA and circulating tumor-derived cfDNA (ctDNA). When ctDNA levels are high, plasma genotyping is technically similar to tumor tissue genotyping; when tumor DNA is absent, cfDNA genotyping is technically more similar to germline genotyping. In practice, cfDNA levels are typically between these extremes, which could theoretically allow for the segregation of somatic and germline cfDNA variants. We hypothesized that genomic analysis of plasma cfDNA could permit simultaneous tumor and germline genotyping, with accurate resolution of tumor-derived variants and germline variants.

To address this question, we chose to study somatic and germline variants in the \textit{EGFR} gene, which include known germline variants and a well described group of oncogenic mutations present in 10%–20% of NSCLC patients. One \textit{EGFR} mutation,
the family history of lung cancer raised suspicion that the T790M mutation might have represented a germline risk allele.

Clinical activity against both T790M has become common in patients with resistance to TKI, osimertinib (7). Because of the challenges of repeat biopsies therapy exhibit exquisite sensitivity to a third-generation EGFR lung cancers harboring T790M-mediated resistance after initial presented with metastatic lung adenocarcinoma; tissue genotyp-

Case report
A 49-year-old never smoker with a family history of lung cancer presented with metastatic lung adenocarcinoma; tissue genotyping showed EGFR L858R and T790M mutations. Given its pre-

Materials and Methods

Protection of human subjects
Patients at DFCI were enrolled to an institutional review board (IRB) approved correlative study allowing plasma collection for genomic analysis. Guardant Health separately received IRB approval for analysis of deidentified data for research purposes; clinical data such as age, stage, and diagnosis was submitted at time of testing on a requisition form. An exemption from IRB review was obtained to study deidentified plasma NGS results provided to DFCI by Guardant Health.

Droplet digital PCR

Blood (6–10 mL) was collected into EDTA lavender capped vacutainer tubes and centrifuged for 10 minutes at 1,200 × g. The plasma supernatant was further cleared by centrifugation for 10 minutes at 3,000 × g. The second supernatant was stored in cryostat tubes at −80°C until use. Cell-free DNA was isolated using the QIAmp Circulating Nucleic Acid Kit (Qiagen) according to manufacturer’s protocol. Droplet digital PCR (ddPCR) was performed as previously described (11). Briefly, TaqMan PCR reaction mixtures were assembled from a 2 × ddPCR Mastermix (Bio-Rad) and custom 40x TaqMan probes/primer sequences made specific for each assay. Droplets were generated using an automated droplet Generator (Bio-Rad). PCR was performed to endpoint. After PCR, droplets were read on either a QX-100 or QX200 droplet reader (Bio-Rad). Analysis of the ddPCR data was performed with QuantaSoft analysis software (Bio-Rad). All ddPCR reagents were ordered from Bio-Rad. All primer and probes for were custom-ordered from Life Technologies.

PCR conditions.
• EGFR L858R. Forward primer, 5’-GGACAGATGCAAGATCAC-GAGTT-3’, reverse primer, 5’-CCCTCCTCTCTATGTATTTCT-TCT-3’; probe sequences: 5’-VIC-AGTTGCGCCACCCCTTAA-

Plasma next-generation sequencing
All plasma sequencing was done using Guardant360 v2.9 (Guardant Health), as described previously (12), as part of routine clinical patient care or through a dedicated research study. Briefly, cfDNA was isolated from 10 mL of whole blood drawn in Streck cell-free DNA tubes, enriched by hybrid capture targeting exons of 70 genes and critical introns of six genes, and sequenced to an average depth of approximately 15,000× on an Illumina NextSeq500. Variant identification and origin assignment were done as per standard clinical testing operations.

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Translational Relevance

Although genomic analysis of plasma cell-free DNA (cfDNA) is increasingly performed to gain insight into cancer biology, cfDNA remains a poorly understood biospecimen made up of DNA from multiple sources. Here, we develop methods for differentiating germline and somatic variants within plasma cfDNA, using germline and somatic EGFR mutations as a clinical model. We then develop and validate a bioinformatic approach permitting us to identify and study incidental germline EGFR T790M mutations within a database of plasma next-generation sequencing results. Our approach suggests cfDNA genomics, currently used for somatic cancer genotyping, may also offer an untapped opportunity for germline genomic research.

T790M, can be detected rarely as a germline variant where its presence has been associated with familial lung cancer (5). Families harboring germline EGFR T790M mutations are currently under investigation at our center as part of an ongoing multicenter clinical trial (NCT01754025; ref. 6). EGFR T790M is more commonly seen as an acquired somatic mutation after a patient with NSCLC develops resistance to EGFR tyrosine kinase inhibi-

EGFR mutations are currently seen as an acquired somatic mutation after a patient with NSCLC develops resistance to EGFR tyrosine kinase inhibitors (TKI). Testing for EGFR T790M has become routine because lung cancers harboring T790M-mediated resistance after initial therapy exhibit exquisite sensitivity to a third-generation EGFR TKI, osimertinib (7). Because of the challenges of repeat biopsies in patients with advanced NSCLC, plasma genotyping to test for T790M has become common in patients with resistance to first-generation EGFR TKIs, and this is an area of active investigation at our center (8). The co-existence of two research programs studying both germline T790M and plasma genotyping for acquired T790M created an opportunity to study the behavior of these germline variants in plasma cfDNA.

Case report

A 49-year-old never smoker with a family history of lung cancer presented with metastatic lung adenocarcinoma; tissue genotyping showed EGFR L858R and T790M mutations (9), first-line afatinib was started; however, the patient returned with progressive brain metastases after only 2 months of therapy. Plasma NGS then demonstrated the previously observed EGFR L858R at an allelic fraction (AF) of 5.3%, whereas the EGFR T790M allele was detected at 50.9% AF (Supplementary Fig. S1A). The patient was initiated on osimertinib, an EGFR TKI active in the setting of EGFR T790M-mediated resistance to initial EGFR TKI (7), and had clinical benefit lasting 9 months, at which time scans showed early progression in the lung. Repeat plasma NGS showed a reduced EGFR L858R at 0.6% AF but T790M relatively stable at 49.2% AF (Supplementary Fig. S1B). After further disease progression, repeat NGS demonstrated increased levels of EGFR L858R at 18% AF, T790M at 54% AF, and a third EGFR mutation that mediates acquired resistance to osimertinib, C797S, at 1.3% AF (10). The presence of an EGFR T790M mutation at initial diagnosis, its high AF at cfDNA analysis, and the family history of lung cancer raised suspicion that the EGFR T790M mutation might have represented a germline risk allele.

Droplet digital PCR

Blood (6–10 mL) was collected into EDTA lavender capped vacutainer tubes and centrifuged for 10 minutes at 1,200 × g. The plasma supernatant was further cleared by centrifugation for 10 minutes at 3,000 × g. The second supernatant was stored in cryostat tubes at −80°C until use. Cell-free DNA was isolated using the QIAmp Circulating Nucleic Acid Kit (Qiagen) according to manufacturer’s protocol. Droplet digital PCR (ddPCR) was performed as previously described (11). Briefly, TaqMan PCR reaction mixtures were assembled from a 2 × ddPCR Mastermix (Bio-Rad) and custom 40x TaqMan probes/primer sequences made specific for each assay. Droplets were generated using an automated droplet Generator (Bio-Rad). PCR was performed to endpoint. After PCR, droplets were read on either a QX-100 or QX200 droplet reader (Bio-Rad). Analysis of the ddPCR data was performed with QuantaSoft analysis software (Bio-Rad). All ddPCR reagents were ordered from Bio-Rad. All primer and probes for were custom-ordered from Life Technologies.

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Germline sequencing

For selected cases, deidentified buffy coat specimens from Guardant were provided to DFCI. With IRB approval, genomic DNA was extracted for Sanger sequencing of EGFR at the Laboratory for Molecular Medicine (LMM) at Harvard, using a sample ID that cannot be linked to the original participant in any way.

Statistical analysis

The relationship between AF of EGFR driver mutation and measures of copy number variation within the heterozygous group of variants was analyzed using a linear regression. The probability density function of the distribution of the standard deviation and mean AFs for individual cases was estimated using a Gaussian approximation, and outliers were identified using the Tukey method (13). Wilson/Brown method was used to determine the 95% confidence interval for EGFR T790M prevalence in each diagnosis of interest (14). The prevalence among the different diagnoses was compared using a two-tailed Fisher exact test.

Results

Plasma ddPCR of EGFR T790M reveals different behaviors of somatic and germline variants in cfDNA

We first studied 85 patients with advanced NSCLC who had an EGFR T790M mutation detected in plasma cfDNA using our previously described ddPCR platform (15). Of these patients, three were known to carry a germline EGFR T790M mutation based on prior germline sequencing, whereas the remainder had acquired EGFR T790M after TKI treatment. Studying the absolute concentration of T790M alleles in copies/mL of plasma, we found that some cases with somatic T790M carried an even higher concentration of mutant T790M alleles in plasma than the three cases with germline EGFR T790M (Fig. 1A). In contrast, when we looked at the AF of T790M, calculated as the proportion of copies of mutant T790M out of all mutant or wild-type variants at that locus, the AF of the three germline cases hovered around 50%, higher than the AF of the somatic T790M cases (Fig. 1A). We then studied changes in the levels of somatic versus germline EGFR mutations in plasma cfDNA upon treatment. In patients carrying acquired EGFR T790M after first-generation TKI resistance, the concentration of both the EGFR T790M mutations and the driver mutations (e.g., L858R or exon 19 deletion) decreased dramatically in response to therapy (Fig. 1B). In contrast, in patients with germline EGFR T790M mutations, their EGFR driver mutation responded on therapy while the EGFR T790M levels stayed relatively stable (Fig. 1B). These data provided proof-of-concept that quantitation of variant levels in plasma cfDNA can potentially be used to discriminate between somatic and germline origins of tumor-associated mutations.

Figure 1.

Germline and tumor-derived EGFR mutations within plasma cell-free DNA. A, Across ddPCR results for 85 patients, germline T790M mutations (green) are present at a similar concentration as somatic T790M mutations (gray), but at a higher allelic fraction (AF). B, Studying ddPCR results for four patients on treatment, each represented by a different color, the concentrations of somatic EGFR mutations decrease while the concentration of germline EGFR T790M remains constant. C, Across plasma NGS results for 950 cases, the AF distribution for EGFR T790M (green) includes a somatic peak also seen with EGFR driver mutations (blue), as well as a heterozygous peak (arrow) also seen more clearly with a common SNP (EGFR Q787Q, gold).
Plasma NGS identifies three populations of variants detected within cfDNA

Although quantitative plasma genotyping is feasible with ddPCR, it is difficult to multiplex PCR assays across multiple genes. In contrast, next-generation sequencing (NGS) has the potential to capture a broad range of variants across a number of cancer-related genes. To further investigate the behavior of germline and somatic EGFR mutations in plasma cfDNA, we utilized the Guardant360 version 2.9 plasma NGS assay (Guardant Health Inc.; Supplementary Table S1). This assay sequences exonic regions of 70 oncogenes and tumor suppressor genes, and intronic regions of six genes in which oncogenic rearrangements occur (12). We first queried the Guardant database of clinical plasma NGS results to study the distribution of somatic and germline EGFR mutations. We identified test sets of 950 consecutive NSCLC samples for each of the following known somatic mutations (L858R and exon 19 deletions), a common germline SNP within the EGFR tyrosine kinase domain (Q787Q; ref. 16), and T790M, and plotted the AF distribution of each (Fig. 1C). The distribution of the known SNP comprised two discrete, normally distributed probability distributions centered at AFs of 50% and 100%, compatible with heterozygosity and homozygosity of the Q787Q allele. The distribution of the known somatic alterations, L858R and exon 19 deletions, in contrast, demonstrated an exponential decay distribution beginning at the assay’s limit of detection (Fig. 1A). Although AF of the driver mutation is not a perfect measure of tumor content in cfDNA (due to the presence or absence of EGFR gene amplification in some cases), it serves as a rough estimate of tumor content in cfDNA across a cohort (17). Studying the distribution of variant AFs in the heterozygous group, which we designated as all variants with an AF between 25% and 75%, we found that the distribution changed as the AF of the EGFR driver mutation increased. An increase in EGFR driver AF was associated with an increased standard deviation of the heterozygous group (Fig. 3B) as well as an increase in the absolute difference between the case and the population mean, both of which suggest the presence of cancer-derived copy number variation. Studying the standard deviation of the AF of the variants in the heterozygous group, we fit a normal distribution to 94 cases whereas 11 cases had outlier characteristics (Supplementary Fig. S2A). Similarly, studying the median of the AF for the variants in the heterozygous group, we fit a normal distribution to 94 cases whereas 11 cases had outlier characteristics (Supplementary Fig. S2B). Because these outlier populations overlapped, 16 cases in total exhibited one of these two outlier characteristics with evidence of high copy number variation in cfDNA, thought to be due to high levels of tumor DNA causing variability in the AF of germline variants.

Increased AF of driver mutation in cfDNA is associated with increased copy number variation in heterozygous variants

To further study the relationship between tumor content in cfDNA and heterozygous copy number variation, we queried the Guardant database for an additional 63 plasma NGS cases which were positive for EGFR T790M and 39 plasma NGS cases positive for an EGFR driver mutation without T790M. These 105 cases each had a median of 107 coding and noncoding variants detected. Looking at the AF distribution of all 10,702 variants in total (Fig. 2B), one can clearly identify a trimodal distribution with three AF peaks at approximately 0%, 49%, and 100%. Compared with noncoding exonic and intronic variants, coding missense and nonsense variants were enriched in the low AF group of variants (Fig. 2C), consistent with this representing a group of cancer-derived variants.

We further studied AF distribution by performing plasma NGS on pretreatment and on-treatment plasma specimens from three cases with germline EGFR T790M known to harbor EGFR driver mutations in their cancer (two with L858R, one with L858Q). Studying the AFs of all coding and noncoding variants identified on plasma NGS, we were able to clearly visualize three groups of variants (Fig. 2A). The lower AF group of variants includes the EGFR driver and TP53 mutations, representing cancer-derived variants. The highest AF group of variants was centered around 100% AF, representing homozygous germline variants. Finally, a middle group of variants centered around 50%, includes the known germline EGFR T790M mutations and represents heterozygous germline variants. On treatment with a third-generation EGFR TKI (two with osimertinib, one with AP27823), the low AF cancer-derived variants decreased or became undetectable (24%–0.2%, 3.7%–ND, 1.1%–ND). In contrast, the middle group of heterozygous germline variants changed only modestly and remained centered around 50% AF (56%–49%, 52%–49%, 49%–50%). Interestingly, some of these heterozygous variants appeared to have an increase in AF as the cancer responded to therapy whereas others had a decrease in AF. We hypothesized that these changes in the heterozygous group on treatment could represent a reduction in tumor-derived copy number variation, leading to a change in the variant AF in cfDNA.

We then similarly studied all coding and noncoding variants from plasma NGS of the initial case presented above (Supplementary Fig. S1A). This revealed a pattern similar to the germline EGFR T790M cases studied, where the patient’s EGFR T790M mutation fell within the heterozygous group of variants, and the AF changed minimally on therapy as compared to the EGFR L858R mutation (Supplementary Fig. S1B). This pattern seemed suspicious for an incidentally detected germline EGFR T790M mutation detected with plasma NGS.
Derived variants with AFs below 30%. We thus propose that by excluding plasma NGS cases with high copy number variation (and thus high tumor content), plasma NGS results can be accurately differentiated into somatic variants within the cancer-derived group and incidentally identified germline risk alleles within the heterozygous group.

Blinded validation of a bioinformatic algorithm for discriminating between germline and somatic alterations in plasma NGS data

Following the logic of these proof-of-concept studies, we developed and evaluated an integrated bioinformatics algorithm to segregate germline and somatic alterations across the 70 genes assayed using plasma NGS. This algorithm first assigns variants a presumed germline or somatic origin using a priori knowledge, including both internal and external databases of known germline and somatic variants (pathogenic and benign). For example, the 

EGFR Q787Q alteration is a benign polymorphism present in ~52% of germline exomes in the ExAC database (http://exac.broadinstitute.org/), allowing this to be designated as of presumed germline origin regardless of allelic fraction (16). Conversely, the 

EGFR L858R alteration is a relatively common oncogenic mutation in NSCLC but does not appear in germline databases, allowing this to be designated as of presumed somatic origin. Such a priori binning usually results in a median of 78 variants per case being assigned as germline, which allows...
construction of a heterozygous probability distribution by variant AF as described in the studies above. If all presumed somatic mutations (which are generally fewer in number) are present below the lower limit of this heterozygous germline distribution, germline-somatic discrimination of the remaining unassigned variants proceeds according to their AF relative to the germline distribution described by a priori variant classification. However, if the AF of presumed somatic variants exceed the AF of the lower limit of the heterozygous germline distribution, or if extreme chromosomal instability is detected (as assessed by the apparent diploid fraction of the genome), germline/somatic discrimination is deemed uncertain for the variants remaining within that region of overlap, and variants are presumed to be somatic in origin and reported as such. This algorithm was intended to allow identification of suspected germline variants with a high positive predictive value, understanding that sensitivity for variants of germline origin will be reduced in settings of high tumor DNA content.

This algorithm was then applied to 21 prospectively collected clinical samples with high AF (30-75%) EGFR T790M mutations detected on plasma NGS (Supplementary Fig. S3). Cases were segregated into two cohorts based upon the germline-somatic segregation of EGFR T790M described above. Cohort A included 11 cases in which the distribution of somatic versus germline derived variants led to the prediction of a germline T790M mutation being present. Cohort B included 10 cases in which determination of germline vs. somatic was complicated by high copy number variation and a broad heterozygous group. The genomic DNA-containing cellular fractions of each sample were then irreversibly deidentified and, with IRB approval, submitted to a CLIA-certified clinical laboratory for EGFR sequencing in a double-blinded manner such that no germline result was traceable to any individual patient. All 11 cases in cohort A were confirmed to harbor a germline EGFR T790M (positive predictive value 100%, 11/11). Of 10 cases in cohort B, one was found to be germline, resulting in a sensitivity of 92% (11/12) and an overall accuracy of 95% (20/21). The presence of a germline sample in Cohort B is suspected to be a case with high tumor content such that the AF of presumed somatic mutations overlapped with heterozygous germline distribution, making it impossible to discriminate germline variants with certainty.
Using a plasma NGS database to study the epidemiology of germline EGFR T790M

Having validated a method for identifying plasma NGS cases which carry germline EGFR T790M, we hypothesized that we could use existing plasma NGS data to learn about the association of germline variants with specific cancer types. We again queried the Guardant clinical testing database of 31,414 consecutive unique patients representing a wide variety of adult solid tumor types to identify 911 cases positive for EGFR T790M, of which 48 were of germline origin as adjudicated by the above methodology. Though nonsquamous NSCLC was the cancer diagnosis in a minority of the overall patient cohort (41%), this was the cancer diagnosis in 43 of the 48 patients with germline EGFR T790M (90%, Fig. 5A). Of the remaining five patients with germline EGFR T790M, three had a related diagnosis (squamous NSCLC, small cell lung cancer, AB 25 50 75 100 Subject Allelic fraction of alterations by plasma NGS (%) EGFR T790M EGFR driver mutation Other coding alterations ND 25 50 75 100 Subject Allelic fraction of alterations by plasma NGS (%) ND Figure 4. Distinguishing heterozygous and tumor-derived coding variants in cases with low copy number variation. In outlier cases with high copy number variation (A), it is difficult to distinguish germline and somatic variants. When there is lower copy number variation (B), it is possible to visually distinguish which cases of germline EGFR T790M (green) are likely germline.

Figure 5.
Prevalence of germline T790M. A, Querying a database of 31,414 unique cancer patients with plasma NGS results, 48 (0.15%) were found to carry a germline EGFR T790M mutation. Nonsquamous non-small cell lung cancer (NSCLC) was the dominant diagnosis in these patients. B, As compared to the population prevalence of germline EGFR T790M in a reference cohort (0.008%), there is a higher prevalence in subjects with nonsquamous NSCLC (0.34%) but not in subjects with other cancers (0.03%, \( P = 0.06 \)), suggesting germline EGFR T790M is a risk variant for lung cancer. *, \( P < 0.05 \)
Discernment of germline and somatic variants in plasma cfDNA has potential impact on the understanding of cancer biology. With tumor NGS, it is difficult to determine if a variant of unknown significance in an oncogene represents a potential driver mutation or a germline polymorphism. In plasma NGS cases without high copy number variation, it now appears possible to differentiate these two types of genomic alterations using a single assay, reducing the risk of a germline polymorphism being therapeutically targeted in error. In addition, in instances where serial plasma genotyping is being used over time to monitor response and resistance to therapy, the ability to distinguish germline and somatic variants in plasma cfDNA can make it easier to accurately track tumor DNA levels. Finally, our approach could aid accurate calculation of tumor mutation burden (TMB), an emerging biomarker for understanding sensitivity and resistance to immune checkpoint inhibitors (29), by reducing the chance of germline polymorphisms being mistaken for potentially antigenic somatic mutations.

One limitation of our analysis is the focus on a single rare germline variant, EGFR T790M; studies of other germline cancer risk alleles are planned. A second limitation is our use of peripheral white cells as our source of germline DNA, an approach which could be vulnerable to somatic mosaicism such as clonal hematopoiesis of indeterminate potential (CHIP; refs. 30, 31). However, the clear majority of mutations found in patients with clonal hematopoiesis are present at an AF in the range of 0% to 30%, and are unlikely to be mistaken for germline variants. As large studies have found no somatic EGFR mutations within peripheral white cells, clonal hematopoiesis would not interfere the analysis performed in this report. Additional validation studies could consider using an alternate source for germline DNA.

Our data highlights the need for laboratories offering plasma genotyping to be vigilant for germline variants and prepared to report to providers when suspicion for a pathogenic germline mutation is high. Moreover, clinicians ordering plasma genotyping must be prepared for the possibility of incidentally identifying germline risk alleles, and will need referral systems in place to care for these patients should such results be identified. Prospective studies are needed to better understand the frequency of such incidental germline findings in routine oncology care.

Disclosure of Potential Conflicts of Interest
J.I. Odegaard, S.R. Fairclough, R.J. Nagy, and R.B. Lanman are employees of Guardant Health; R.J. Nagy and R.B. Lanman own ownership interest in Guardant Health. J.E. Garber receives research funding or has research collaborations with Myriad Genetics, AstraZeneca, and Ambrus and receives consulting fees from Helix. C.P. Paweletz received consulting fees from Digital Bioanalytics and honoraria from Bio-Rad; G.R. Oxnard received consulting fees from AstraZeneca and Invivata and honoraria from Bio-Rad and Symex. All other authors have no potential conflicts of interest to report.

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