Amplicon-based next-generation sequencing of plasma cell-free DNA for detection of driver and resistance mutations in advanced non-small cell lung cancer

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We report compelling accuracy using tagged amplicon sequencing for the detection of a full range of genotypes, including fusion genes, in cell-free DNA from advanced NSCLC patients. This alternative to hybrid-capture approaches has the ability for early detection of diverse mechanisms of resistance to EGFR-TKIs, including acquired resistance mutations and amplifications.

Abstract

Background: Genomic analysis of plasma cell-free DNA is transforming lung cancer care, however available assays are limited by cost, turnaround time, and imperfect accuracy. Here we study amplicon-based plasma next-generation sequencing (NGS), rather than hybrid-capture-based plasma NGS, hypothesizing this would allow sensitive detection and monitoring of driver and resistance mutations in advanced non-small cell lung cancer (NSCLC).

Methods: Plasma samples from patients with NSCLC and a known targetable genotype (*EGFR*, *ALK/ROS1* and other rare genotypes) were collected while on therapy and analyzed, blinded to tumor genotype. Plasma NGS was performed using enhanced tagged amplicon sequencing of hotspots and coding regions from 36 genes, as well as intronic coverage for detection of *ALK*/*ROS1* fusions. Diagnostic accuracy was compared to plasma ddPCR and tumor genotype.

Results: A total of 168 specimens from 46 patients were studied. Matched plasma NGS and ddPCR across 120 variants from 80 samples revealed high concordance of allelic fraction (R^2=0.95). Pretreatment, sensitivity of plasma NGS for the detection of *EGFR* driver mutations was 100% (30/30), compared to 87% for ddPCR (26/30). A full spectrum of rare driver oncogenic mutations could be detected including sensitive detection of *ALK*/*ROS1* fusions (8/9 detected, 89%). Studying 25 patients positive for *EGFR* T790M that developed resistance to osimertinib, 15 resistance mechanisms could be detected including tertiary *EGFR* mutations (C797S, Q791P) and mutations or amplifications of non-*EGFR* genes, some of which could be detected pre-treatment or months before progression.

Conclusions: This blinded analysis demonstrates the ability of amplicon-based plasma NGS to detect a full range of targetable genotypes in NSCLC, including fusion genes, with high accuracy. The ability of plasma NGS to detect a range of pre-existing and acquired resistance mechanisms highlights its potential value as an alternative to single mutation digital PCR-based plasma assays for personalizing treatment of TKI resistance in lung cancer.

Key words: Amplicon-based next generation sequencing, plasma genotyping, circulating tumor DNA, resistance mechanisms, fusion genes, *EGFR*

Introduction

Genotype-directed treatment of non-small cell lung cancer (NSCLC) has led to dramatic improvement in the management of selected patients harboring a targetable oncogenic driver [1]. The limited availability of tissue to test an increasing number of potentially actionable genotypes and a better understanding of druggable mechanisms of resistance [2] have created a need for a rapid, repeatable and non-invasive access to the tumor biology throughout treatment. Genotyping of plasma cell-free DNA (cfDNA) is already an established diagnostic tool that can guide rapid initiation of TKI therapy in *EGFR*-mutant NSCLC [3,4], avoiding some invasive biopsies. However, the most established assays are digital PCR-based, detecting mutations at only a single site in a predefined gene.

Unlike digital PCR plasma genotyping, next generation sequencing (NGS) of cfDNA has the potential to more broadly assess the molecular profile of the tumor. Hybrid capture-based NGS of plasma cfDNA has already been well evaluated in NSCLC [5-7]. While this technical approach permits sequencing of dozens of genes and detection of complex variants, including rearrangements, concordance with matched tumor genotyping has been suboptimal in some series [5,6]. Amplicon-based NGS is a well-established alternate technology, which uses target gene enrichment by PCR with a set of primers for exons or hotspots of selected genes [8], and is the basis for the tumor NGS assay that recently received approval by the US FDA (Oncomine™ Dx Target, ThermoFisher). While this technology is less well studied for NGS of cfDNA, where the levels of input DNA (and tumor fraction) are usually very low, we hypothesized barcoded amplicon-based NGS would provide excellent sensitivity with limited sequencing artifact, and would represent a compelling alternative to hybrid capture-based plasma NGS.

Methods

Patients were identified with stage IIIB/IV, progressive NSCLC harboring a known tumor genotype and consented for plasma collection as part of two ongoing correlative studies at our institution. Plasma was collected for analysis prior to receipt of targeted therapy; when feasible, plasma was also collected at the initial toxicity evaluation and with restaging scans until development of resistance. We first studied a cohort of 30 patients with *EGFR*-mutant NSCLC and

T790M-positive resistance receiving osimertinib; upon successful proof of principle, we then additionally studied a cohort of 16 patients harboring other rare targetable genotypes, totaling 168 time points from 46 subjects. Plasma analyses were performed blinded to clinical information such as tumor genotype. Sensitivity and specificity for plasma ddPCR and plasma NGS assays were calculated using clinically performed tumor genotyping as reference standard; tumor genotyping was performed using hybrid-capture NGS whenever possible [9]. Concordance of variant allelic fraction (AF) between plasma ddPCR and NGS was calculated using Kendall concordance coefficient.

Droplet digital PCR (ddPCR)

Plasma genotyping using ddPCR was performed for all cases with *EGFR*-mutant NSCLC, as well as to validate selected non-*EGFR* hotspot mutations based upon assay availability (e.g. *KRAS*, *PIK3CA*, *BRAF* mutations). ddPCR was performed at the Belfer Center for Applied Cancer Science as described previously [3]. Remaining aliquots of plasma were allocated for plasma NGS, requiring a minimum of 1-2 ml of plasma or a corresponding quantity of extracted cfDNA.

Plasma NGS

Amplicon-based plasma NGS was performed by Inivata (Morrisville, NC), using InVision^{TM}, an enhanced version of TAm-Seq technology, based on methods previously described [10-12]. 36 cancer-related genes were sequenced using gene specific primers designed to hotspots and entire coding regions of interest **(Supplementary Figure 1)**. Extracted cfDNA is first quantified by digital PCR targeting a 108bp region of the ribonuclease P/MRP subunit p30 (*RPP30*) gene [13]. Next generation sequencing libraries are then prepared from 2,000 - 16,000 amplifiable copies of the genome (~6.6ng to 53 ng of amplifiable DNA) using a two-step PCR amplification process incorporating replicate and patient-specific barcodes and Illumina sequencing adaptors. In the first step PCR reaction, amplicons ranging from 73bp to 155bp are generated which were designed and optimized for the DNA fragment size found in circulation. Each sample is analysed multiple times allowing the identification of false positive and true positive calls [10,12]. After further clean-up using SPRI beads, samples are quantified and pooled to generate a normalized library of 12 nM. 1.8 pM libraries are sequenced on the Illumina NextSeq with 5% PhiX added to monitor sequencing performance. A minimum Phred quality score of 30 for each base was required for inclusion in the analytics. Sequencing files were analysed using Invata's proprietary Somatic Mutation Analysis (ISoMA) pipeline.

In a subset of cases known to harbour oncogenic fusions, a separate aliquot of plasma cfDNA was tested using a novel technology designed to identify *ALK* and *ROS1* breakpoints. The novel PCR based assay has been designed to capture all major *EML4-ALK* variants in NSCLC, encompassing 95% of variants found in COSMIC (version 78). The panel also captures 90% of ROS1 fusions in NSCLC as described in the COSMIC database and identifies the breakpoints occurring between *CD74-ROS1*, *SLC34A2-ROS1*, *SDC4-ROS1* and *EZR-ROS1*. The ALK and ROS1 assay covers approximately 50 kb of intronic and exonic sequences, allowing the identification of precise DNA breakpoints in regions that are frequently re-arranged. Libraries were prepared and sequenced on the Illumina NextSeq 500 as described above.

Sequencing files were analysed using Inivata's proprietary FUSP pipeline, which identifies specific DNA sequences brought together creating the fusions outlined above.

Results

Detecting known EGFR mutations

Using tissue genotyping as a reference standard, and ddPCR for orthogonal validation, sensitivity of plasma NGS was analyzed across 30 cases with *EGFR*-mutant NSCLC and acquired T790M. Sensitivity for detection of the driver *EGFR* mutation was 100% (30/30) with plasma NGS and 87% (26/30) for plasma ddPCR (p=0.11; 10/11 L858R, 16/19 exon 19 deletion). Sensitivity for the detection of T790M was 77% (23/30) for plasma NGS and 80% (24/30) for ddPCR. Discordance was only seen at low AF, below 1.1% (**Figure 1A**), where plasma NGS detected 4 driver mutations missed with ddPCR. Two T790M mutations detected with ddPCR and not with NGS were, retrospectively, below our ddPCR threshold for clinical reporting and could have been false positives (**Supplementary Figure 2).** Quantitative concordance of AF between NGS and ddPCR was excellent across 120 variants (from 80 specimens) positive for an *EGFR* mutation with both assays (R^2= 0.95, **Figure 1B**).

Detection of rare variants and fusion genes

Studying 9 cases with known *ALK* or *ROS1* fusions, sensitivity of plasma NGS was 89% (6/7 *EML4-ALK*, 2/2 *CD74-ROS1;* **Figure 1C & 1D**); the one missed *ALK* case was the single patient studied with stage IIIB disease. Studying 6 cases with other mutations in the kinase domain (2 *MET* splice mutation, 3 *BRAF* mutations, 1 *HER2* exon 20 insertion), 4 were correctly identified (**Figure 1C**). Two cases of *BRAF* V600E in stage IV patients were undetectable with plasma NGS, and additionally were found to be undetectable on ddPCR. Interestingly one patient with a *BRAF* V600E mutation on tumor genotyping (RT-PCR) instead had a *KRAS* G12D mutation detected on plasma NGS (0.2% AF); this patient was a heavy smoker who did not respond to BRAF inhibitor therapy.

Specificity across other non-driver variants

To study specificity, we studied 19 cases with tumor NGS available (8 pre-osimertinib, 5 post-osimertinib, 6 pre-treatment specimens with other rare genotypes). For this analysis, we excluded each patient's driver oncogene, to avoid acquired resistance mutations, and limited our analysis to genes covered by both tissue and plasma NGS panels. Composite specificity-by-gene was 99.5% across 665 genes sequenced, with 3 false positives (**Table 1**). First, one *PIK3CA* E545K mutation was found on plasma NGS at low AF (0.6%) but not in tumor; the mutation was confirmed in cfDNA by plasma ddPCR at the same AF (0.6%). Second, three point mutations in *CTNNB1* were found at low AF (S37F 0.3%, S45C 1%, S45F 0.7%) on plasma NGS following osimertinib; corresponding tissue NGS did not reveal these, though tumor content may have been suboptimal (*TP53* H193R mutation was found at 10% in tissue vs. 30% in plasma). Third, an *IDH1* R132H mutation was detected at low AF (0.3%) on plasma NGS but not in the corresponding tumor NGS; this variant recurred at multiple timepoints at a stable AF in this patient's cfDNA, suspicious for clonal hematopoiesis (**Supplementary Figure 3**) [14].

Detection of resistance mechanisms using plasma NGS

We studied resistance in 25 osimertinib-treated subjects with detectable *EGFR* driver mutations with plasma NGS at the time of resistance. 15 patients (60%) lost T790M at resistance (**Table 2**), and 4 of them had a non-*EGFR* resistance mutation identified: one *PIK3CA* E545K mutation (0.13% AF), two *BRAF* V600E mutations (12.5% AF, 0.4% AF), one *KRAS* G12S mutation (0.2% AF). Gene amplifications were also detected at resistance in 4 patients (**Figure 2**): two *HER2* amplifications (one occurring concomitantly with *BRAF* V600E), one *MET* amplification, and one *FGFR1* amplification. 10 patients maintained *EGFR* T790M at resistance, 8 of whom acquired a tertiary *EGFR* C797S resistance mutation. In 3 patients, two C797S variants (c.2889T>A and c.2390G>C) were present at resistance at different AF (**Table 2**). One of these three patients additionally acquired a novel *EGFR* Q791P mutation (2.6% AF; **Supplementary Figure 4**). This mutation was confirmed in cfDNA using another amplicon-based NGS approach (QIAseq DNA Targeted Lung Panel, Qiagen; 2% AF). Interestingly, two patients with maintained T790M additionally acquired canonical *KRAS* mutations (*KRAS* G13D 0.2% AF; *KRAS* Q61K 2.8% AF).

Resistance genotyping was also piloted in 7 specimens from patients with rare genotypes treated with various TKIs (**Supplementary Table 1**). A mechanism of resistance could only be detected in one *ALK*-positive case (*ALK* C1156Y, 0.55% AF) after treatment with crizotinib. One *ROS1* G2032R acquired resistance mutation, detected in tissue NGS in a *ROS1* case after crizotinib, was not detected in plasma.

Early detection of resistance through serial plasma NGS

At least 3 serial plasma specimens (up to 8) were studied for 25 subjects treated with osimertinib, to pilot the early detection of resistance mechanisms. In four cases, a competing resistance mutation (*KRAS* Q61K, 2 *BRAF V*600E, *PIK3CA* E545K) could be detected pretreatment by plasma NGS. In these four cases, a complete and rapid clearance of the T790M clone was seen without immediate plasma clearance of the driver *EGFR* mutation (**Figure 2**). No cases of *EGFR* C797S could be detected pre-osimertinib, though monitoring multiple timepoints on therapy revealed this mutation can be seen multiple months before clinical progression (**Figure 2**).

Discussion

In this blinded clinical validation, we demonstrate the ability of amplicon-based plasma NGS to sensitively detect a wide range of molecular alterations, including chromosomal rearrangements, in advanced NSCLC. Few prior studies have aimed to clinically validate amplicon-based NGS of cfDNA. Couraud et al tested a 12-gene panel on the IonTorrent platform and found an overall sensitivity of 58% [15]. The first generation of the TamSeq technology was developed by Forshew et al on a dilution series of circulating DNA containing increasing frequencies of a rare allele, using a 48-primer set covering coding regions and hotspots in 6 genes. It was then validated using plasma samples from metastatic ovarian cancer patents. This foundational work reported high sensitivity, specificity and quantitative concordance with digital PCR, but also the ability to follow the subclonal evolution of tumors in a limited number of patients [10]. We confirm in a NSCLC population the high sensitivity of the InVision^{TM} assay, matching (and in some cases exceeding) the sensitivity of plasma ddPCR. This high sensitivity, combined with accurate quantification and an ability to detect a full spectrum of genomic variants (something difficult to do with PCR assays), makes this NGS-based approach a compelling alternative to ddPCR for detection of T790M and other actionable mutations.

We also report for the first time the ability of a novel amplicon-sequencing assay to detect gene fusions, with high sensitivity in cfDNA; the one missed *ALK* case was a patient with stage IIIB disease. In contrast, existing data on hybrid-capture NGS have suggested suboptimal sensitivity (54%) for the detection of *EML4-ALK* fusions [16]. Our team has also previously reported the detection of fusions genes (2/3 *ALK*, 2/3 *RET*, 2/2 *ROS1*) using a bias-corrected, targeted cfDNA NGS, detecting breakpoints and fusions partners using a single assay [7]. Further prospective evaluation is needed to clarify the potential sensitivity advantages of the different plasma NGS approaches for low AF mutations and the full spectrum of targetable fusions.

Though NGS of tumor tissue may be an imperfect reference standard in patients with drug resistance, our data nonetheless suggest compelling specificity with the InVision assay, which is reassuring given the plasma-positive, tumor-negative discordance seen at times with hybrid-capture NGS [6]. We identified four potential false positives – one we confirmed with plasma ddPCR, one is consistent with CHIP, and one we believe is likely due to subclonal resistance heterogeneity [14]. The only concerning false positive was a *KRAS* G12D mutation found at low AF (0.2%), which was not detectable with plasma ddPCR, but consistent with the patient's clinical presentation, and this case highlights the challenges of validating assays which are potentially more sensitive than established validated assays. Still, the lack of any false positives for EGFR driver mutations highlights that targetable mutations, even when found at a very low AF, can be considered actionable when found with a well validated assay.

Our data highlight the potential value of amplicon-based plasma NGS for characterizing treatment resistance in NSCLC. In patients treated with osimertinib, we could detect common mechanisms of resistance to EGFR-TKIs [2], including expected point mutations, expected gene amplifications (*MET*, *HER2*), and a novel tertiary *EGFR* mutation (Q791P), which was cross-validated by another sequencing approach. Surprisingly, we found three low level *KRAS* mutations (one which was confirmed in tumor and has been reported previously), suggesting this may be a recurring mechanism of resistance to osimertinib. Serial plasma NGS also offers potential insights – for example in some cases with an incomplete response in the driver *EGFR* mutation (which has been suggested previously to indicate poorer treatment outcomes [17]), we could detect early presence of a coexistent resistance mutation. The ability of plasma NGS to detect resistance mutations coexistent with T790M as well as detecting a range of tertiary *EGFR* mutations makes it a potentially powerful alternative to established PCR-based assays for resistance genotyping. Furthermore, the detection in a few cases (**Figure 2**) of resistance mutations at low AF that overgrow the T790M on osimertinib therapy supports the potential clinical value of such low AF resistance mutations, and deserves further study.

In conclusion, we demonstrate herein the ability of amplicon-based NGS to detect with a wide range of targetable genotypes in advanced NSCLC, including high accuracy for point mutations and indels, and compelling initial data for gene fusions and CNVs. This approach also permits early detection of resistance mechanisms during treatment, making it a potentially valuable tool to guide early modifications of targeted therapies. Amplicon-based plasma NGS has attractive sensitivity and specificity and deserves further study as an alternative to hybrid-capture approaches.

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Conflict of interest: CP received honoraria form Astra Zeneca, Bio-Rad and Clovis and is part of the Advisory Board of Dropworks. GRO received consulting fees from AstraZeneca, Ariad/Takada, Inivata, and honoraria from Bio-Rad, Chugai, Guardant, and Sysmex. GJ, VP, KH and JB are employees and share-holders of Inivata Ltd. Inivata Ltd commercializes assays based on the technology described in this paper. All other authors have no potential conflicts of interest to report.

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Figure and Table legends:

Table 1: Comparison of 19 cases with matched pretreatment (*n*=14) or post-treatment (*n*=5) tumor NGS. Limited to non-driver variants covered by the NGS panel (sup Fig 1), 3 plasma NGS-positive/tissue NGS-negative discordant results were seen (3/684 genes sequenced, specificity 99.6%)

Table 2: Detection of acquired resistance mechanisms to osimertinib at resistance (limited to patients with a resistance mechanism identified (15/25)). **Top**: 7/15 patients with acquired resistance to osimertinib and loss of T790M had a detectable mechanism of resistance. **Bottom**: 8/10 patients with acquired resistance to osimertinib and maintained T790M had one or multiple mechanisms of resistance to osimertinib, all of them having one or multiple tertiary *EGFR* mutation(s).

Figure 1: A: Focusing on 9 cases of *EGFR*-mutant NSCLC with the lowest tumor DNA shed (<1.1% AF), sensitivity appears better with amplicon-based plasma NGS compared to plasma ddPCR. *Two T790M mutations were detected with ddPCR but not NGS, though the ddPCR signal was below the level for clinical reporting and may have been a false positive (Supplemental Figure 2). (AF= allelic fraction). **B**: Quantitative concordance was high (R^2=0.95) across 120 *EGFR* variants from 80 specimens detected both with plasma NGS and plasma ddPCR. **C:** Detection of a range of fusions and rare genotypes using amplicon-based plasma NGS. One apparent false positive (*) secondarily tested negative by ddPCR for both *KRAS* and *BRAF* mutations. **D**: Three examples where fusion detection permits the determination of fusion partner and breakpoint.

Figure 2: A&B: Acquired gene amplifications detected in plasma cfDNA at the time of resistance to osimertinib. **A**: Acquired *ERBB2* and *MYC* amplification (driver AF 63%). **B**: Acquired *ERBB2*

amplification and *TP53* deletion (driver AF 38%). **C**: *EGFR* amplification and acquired *MET* amplification (driver AF 22%). **D-G**: Early detection of mechanisms of resistance to osimertinib through serial NGS of cfDNA in 4 patients. **D and E**: Two cases with mixed response in plasma (complete clearance of T790M, uncomplete response of the driver) had loss of T790M at the time of resistance. Possible competing resistance mutations (**D**: *BRAF* V600E; **E**: *PIK3CA* E545K) coexisting with T790M at baseline can be detected pretreatment. **F and G**: In two patients with maintained T790M at resistance, serial plasma NGS can detect acquired tertiary *EGFR* mutations several months before clinical progression (**F**: 3 months; **G**: 7.5 months). In one patient (**F**), plasma NGS could also detect a competing *KRAS* Q61K mutation at low AF at baseline that with increased AF at resistance.

Supplementary Figure 1: List of the 36 genes covered by the Inivata NGS panel.

Supplementary Figure 2 : ddPCR plots from two patients reported positive for T790M mutation with ddPCR and not NGS. Retrospective analysis of the ddPCR files makes these 2 cases suspicious for false positive ddPCR calls (2 positive droplets, below the threshold for clinical report).

Supplementary Figure 3 : Detection of a *IDH1* mutation at low and constant AF, not following the same kinetics of other tumor-derived mutations, suggests a hematopoietic origin (e.g. clonal hematopoiesis of indeterminate potential, CHIP).

Supplementary Figure 4: BAM file showing sequencing reads selected for *EGFR* T790M mutation (AF 3.6%), showing the detection of the novel tertiary *EGFR* Q791P mutation (AF 2.6%) as well as two C797S mutation (G_C, AF 1%; T_A, AF 2.7%).

Supplementary Table 1: Resistance plasma NGS from 7 patients harboring a rare genotype were analyzed. An ALK resistance C1156Y mutation was detected at the time of resistance to crizotinib in one patient. One *ROS1* G2032R acquired resistant mutation was detected in tissue but not in plasma.

Driver	Pre-TKI Tissue NGS	Pre-TKI Plasma NGS
EGFR	TP53 A161T (55%)	none
EGFR	TP53 R110L (56%)	TP53 R110L (7.5%)
EGFR	TP53 166_167GA>A (64%)	TP53 frameshift (2.3 %)
EGFR	none	none
EGFR	TP53 F134L (37%)	TP53 F134L (6.56%)
EGFR	none	**PIK3CA E545K (0.6%)**
EGFR	TP53 L43* (32%)	TP53 L43 (7.97%)
EGFR	TP53 C242F (76%)	TP53 C242F (7.78%)
BRAF	STK11 D343N (40%)	STK11 D343N (50%)
MET	TP53 R248W	TP53 R248W (0.6%)
ROS1	none	none
ROS1	none	none
ALK	none	none
ALK	TP53 R337C	TP53 R337C (1.2%)
	Post-TKI Tissue NGS	Post-TKI Plasma NGS
EGFR	TP53 R282W (44%)	TP53 R282W (27%)
EGFR	KRAS Q61K (30%) TP53 Q104* (53%)	KRAS Q61K (2.8%) TP53 Q104* (12%)
EGFR	none	none
EGFR	TP53 H193R (MAF 10%)	TP53 H193R (27%) **CTNNB1 (S37F 0.3%, S45C 1%, S45F 0.7%)**
EGFR	TP53 del (MAF 53%)	TP53 frameshift (MAF 1.21%) **IDH1 R132H (0.33%)**

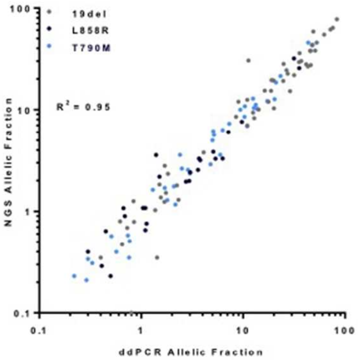
Driver at resistance	T790M at resistance	Resistance, post-osimertinib NGS (beside *EGFR* driver and T790M)
Del19 63.4%	Lost	***BRAF*** V600E 12.4% ***HER2*** amp
Del19 2.4%	Lost	***BRAF*** V600E 0.4%
Del19 19%	Lost	***PIK3CA*** E545K 1.2%
Del19 12%	Lost	***MET*** amp
Del19 37.9%	Lost	***HER2*** amp
Del19 1.8%	Lost	***FGFR1*** amp
L858R 0.21%	Lost	***KRAS*** G12S 0.2%
Del19 43%	46.4%	***EGFR*** C797S 26.6%
Del19 15.2%	11%	***EGFR*** C797S 7.9% ***KRAS*** Q61K 2.8%
Del19 27.4%	11.2%	***EGFR*** C797S 0.4% ***PTEN*** Y27C 21.9% ***KRAS*** G13D 0.25%
L858R 7.6%	7.3%	***EGFR*** C797S T_A 1.3% ***EGFR*** C797S G_C 1.1%
L858R 3.2%	3.6%	***EGFR*** C797S T_A 2.7% ***EGFR*** C797S G_C 1% ***EGFR*** Q791P A_C 2.6%
del19 37.7%	18.3%	***BRAF*** V600E 0.4% ***EGFR*** C797S T_A 13.5%
del19 12%	7%	***EGFR*** C797S T_A 2.9%
del19 31.6%	9.2%	***EGFR*** C797S T_A 6.2% ***EGFR*** C797S G_C 0.9%

Genotype	Treatment	Resistance tissue NGS	Resistance plasma NGS
ALK/EML4	crizotinib	NA	***ALK*** **C1156Y**
ALK/EML4	alectinib	none	no mechanism of resistance
ALK/EML4	crizotinib	NA	no mechanism of resistance
HER2	carboplatin-pemetrexed	NA	no mechanism of resistance
ROS1	crizotinib	*ROS1* G2032R	**no mechanism of resistance**
ROS1	crizotinib	NA	no mechanism of resistance

1A

Tissue genotype	Driver (MAF)		T790M (MAF)	
	ddPCR	NGS	ddPCR	NGS
L858R + T790M	1.1	0.65	0	0.28
L858R + T790M	0.7	1	0.45*	0
Del19 + T790M	0.65	0.47	0	0
L858R + T790M	0.5	0.23	0.4	0.57
L858R + T790M	0	0.057	0	0
Del19 + T790M	0	0.2	0.35*	0
Del19 + T790M	0	0.05	0	0
Del19 + T790M	0	0.9	0	0
Del19 + T790M	0.4	0.35	0	0
Low AF Sensitivity (n=9)	55%	100%	33%	22%

1B



1C

Tissue Genotype	Plasma NGS
BRAF V600E	***KRAS* G12D (0.18%)***
BRAF V600E	none
BRAF G469A	*BRAF* G469A (12.8%)
MET exon 14	*MET* exon 14 (1.4%)
MET exon 14	MET exon 14 (12.1%)
HER2 exon 20 ins	*HER2* exon 20 ins (0.3%)
Sensitivity (rare SNVs)	66.7% (4/6)
EML4-ALK fusion	*EML4-ALK* fusion
EML4-ALK fusion	*EML4-ALK* fusion
EML4-ALK fusion	*EML4-ALK* fusion
EML4-ALK fusion	*EML4-ALK* fusion
EML4-ALK fusion	*EML4-ALK* fusion
EML4-ALK fusion	*EML4-ALK* fusion
EML4-ALK fusion	**none**
CD74-ROS1 fusion	*CD74-ROS1* fusion
CD74-ROS1 fusion	*CD74-ROS1* fusion
Sensitivity (*ALK/ROS1*)	89% (8/9)

1D

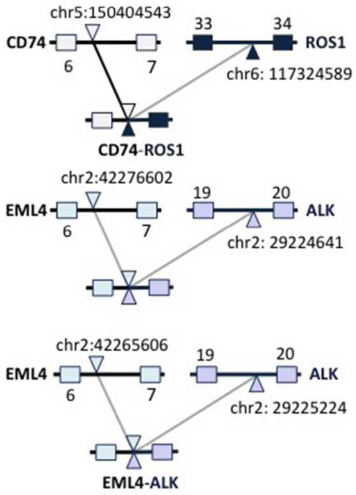


Figure 1: A: Focusing on 9 cases of EGFR-mutant NSCLC with the lowest tumor DNA shed (<1.1% AF), sensitivity appears better with amplicon-based plasma NGS compared to plasma ddPCR. *Two T790M mutations were detected with ddPCR but not NGS, though the ddPCR signal was below the level for clinical reporting and may have been a false positive (Supplemental Figure 2). (AF= allelic fraction). B: Quantitative concordance was high (R2=0.95) across 120 EGFR variants from 80 specimens detected both with plasma NGS and plasma ddPCR. C: Detection of a range of fusions and rare genotypes using amplicon-based plasma NGS. One apparent false positive (*) secondarily tested negative by ddPCR for both KRAS and BRAF mutations. D: Three examples where fusion detection permits the determination of fusion partner and breakpoint.

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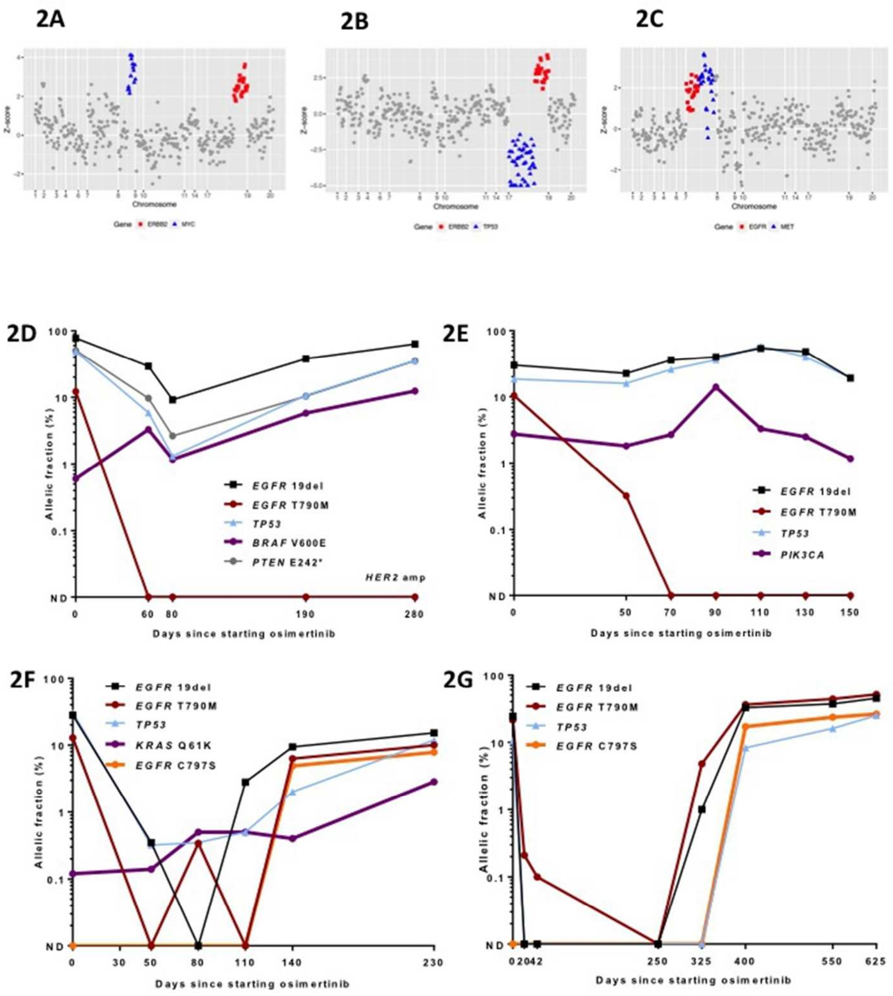

Figure 2: A&B: Acquired gene amplifications detected in plasma cfDNA at the time of resistance to osimertinib. A: Acquired ERBB2 and MYC amplification (driver AF 63%). B: Acquired ERBB2 amplification and TP53 deletion (driver AF 38%). C: EGFR amplification and acquired MET amplification (driver AF 22%). D-G: Early detection of mechanisms of resistance to osimertinib through serial NGS of cfDNA in 4 patients. D and E: Two cases with mixed response in plasma (complete clearance of T790M, uncomplete response of the driver) had loss of T790M at the time of resistance. Possible competing resistance mutations (D: BRAF V600E; E: PIK3CA E545K) coexisting with T790M at baseline can be detected pretreatment. F and G: In two patients with maintained T790M at resistance, serial plasma NGS can detect acquired tertiary EGFR mutations several months before clinical progression (F: 3 months; G: 7.5 months). In one patient (F), plasma NGS could also detect a competing KRAS Q61K mutation at low AF at baseline that with increased AF at resistance.

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