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Sensitivity of next-generation sequencing assays detecting oncogenic fusions in plasma cell-free DNA

Julianna G. Supplee[a], Marina S.D. Milan[b], Lee P. Lim[c], Kristy T. Potts[c], Lynette M. Sholl[d], Geoffrey R. Oxnard[b], Cloud P. Paweletz[a,*]

[a] Belfer Center for Applied Cancer Science, Dana-Farber Cancer Institute, 360 Longwood Avenue, Boston, MA, 02215, USA
[b] Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA, 02215, USA
[c] Resolution Bioscience, Inc., 550 Kirkland Way, Kirkland, WA, 98033, USA
[d] Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA, 02115, USA

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ABSTRACT

Objectives: Plasma genotyping represents an opportunity for convenient detection of clinically actionable mutations in advanced cancer patients, such has been well-documented in non-small cell lung cancer (NSCLC). Oncogenic gene fusions are complex variants that may be more challenging to detect by next-generation sequencing (NGS) of plasma cell-free DNA (cfDNA). Rigorous evaluation of plasma NGS assays in the detection of fusions is needed to maximize clinical utility.

Materials and methods: : Additional plasma was collected from patients with advanced NSCLC and *ALK, ROS1,* or *RET* gene fusions in tissue who had undergone clinical plasma NGS using Guardant360™(G360, Guardant Health). We then sequenced extracted cfDNA with a plasma NGS kit focused on known driver mutations in NSCLC (ctDx-Lung, Resolution Bioscience) with cloud-based bioinformatic analysis and blinded variant calling.

Results: Of 16 patients assayed known to harbor an*ALK, ROS1,* or *RET* in tumor, G360 detected fusions in 7 cases, ctDx-Lung detected fusions in 13 cases, and 3 cases were detected by neither. Of the 7 fusions detected by both assays, G360 reported lower mutant allelic fractions (AF). In cases missed by G360, tumor derived *TP53* mutations were often detected confirming presence of tumor DNA. Raw sequencing data showed that inverted or out-of-frame variants were overrepresented in cases detected using ctDx-Lung but not by G360.

Conclusion: Focusing on complex, clinically actionable mutations using tumor as a reference standard allows for evaluation of technical differences in plasma NGS assays that may impact clinical performance. Noting the heterogeneity of fusion sequences observed in NSCLC, we hypothesize that differences in hybrid capture techniques and bioinformatic calling may be sources of variations in sensitivity among these assays.

1. Introduction

Tumor genotyping has become an integral part of identifying actionable, targetable mutations and administering personalized therapy for patients with advanced non-small cell lung cancer (NSCLC). Non-invasive genotyping assays for plasma cell free DNA (cfDNA) have been adopted broadly for care of advanced NSCLC based on data indicating a high positive predictive value for actionable mutations [1]. Plasma genotyping has the potential to expand access to personalized care for those whose tissue biopsies are inadequate or unavailable, as well as potentially offering an emerging opportunity for cancer monitoring and detection of minimum residual disease [2]. However, the reliability of cfDNA next-generation sequencing (NGS) assays is incompletely characterized [3], as highlighted by recent reports of discordance due to clonal hematopoiesis and germline variant handling [4]. While the source of false negatives has often been attributed to low tumor DNA shed, the role of technical differences between assays as a source of false negatives is less understood. Up to 10% of lung adenocarcinomas can harbor a gene fusion in *ALK*, *ROS1,* or *RET* which can be responsive to tyrosine kinase inhibitor (TKI) therapies, but these mutations are complex genomic events that normally require sophisticated methods such as break-apart FISH or NGS to identify. The focus of the present study was to examine differences between two hybrid-capture NGS assays for detection of these targetable fusions in plasma cfDNA.

* Corresponding author at: Belfer Center for Applied Cancer Science, Dana-Farber Cancer Institute, LC-4218, 360 Brookline Avenue, Boston, MA, USA.
E-mail addresses: julianna_supplee@dfci.harvard.edu (J.G. Supplee), MarinaS_Milan@dfci.harvard.edu (M.S.D. Milan), lplim@resolutionbio.com (L.P. Lim), kristy@resolutionbio.com (K.T. Potts), lmsholl@bwh.harvard.edu (L.M. Sholl), geoffrey_oxnard@dfci.harvard.edu (G.R. Oxnard), cloudp_paweletz@dfci.harvard.edu (C.P. Paweletz).

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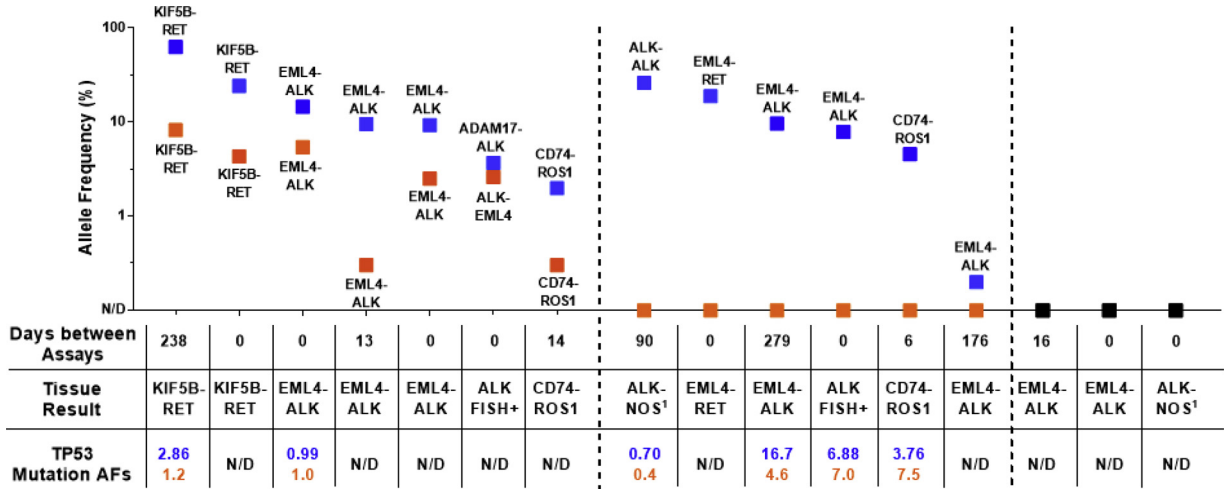

Fig. 1. Discordance between two next-generation sequencing (NGS) assays for plasma cell-free DNA. Gene fusions are shown as detected in plasma by Guardant360 (orange) and by the Resolution Bioscience ctDx-Lung kit (blue), or neither (black). The highest allele frequencies (AF, %) of *TP53* variants detected by both assays, a marker of circulating tumor DNA shed, are listed with matching colors. Of 16 cases total, 7 fusions were detected by G360 (44%) and 13 were detected by the ctDx-Lung kit (81%, p = 0.66 with two-sided Fisher's exact test). [1]Tissue NGS could not successfully map *ALK* fusion partner.

2. Materials and methods

An institutional database of NSCLC patients was queried and identified 169 patients who had undergone testing between April 2016 and June 2018 with the Guardant360 plasma NGS assay (Guardant Health, Redwood City, CA, hereafter referred to as G360), which has previously reported an average coverage of ~5000 unique molecules [5]. Of these, 28 patients were found to have an *ALK*, *ROS1*, or *RET* rearrangement in their tumor through tissue testing (Supplementary Fig. 1). 12 patients were excluded as there was no banked plasma and additional plasma could not be collected. The remaining 16 patients made up our study cohort. Of these, 5 patients had test results by Guardant360 v14.0, 4 by Guardant360 v15.0; 5 by Guardant360 v16.0, and 2 by Guardant360 v17.0. In cases in which paired specimens were not available (69% had plasma collected within two weeks of G360), concurrently reported SNVs, especially in *TP53*, were used in our study to confirm DNA shed by the tumor.

Blood was collected in a purple K_2EDTA tube with IRB approval and patient consent. Plasma was isolated by centrifugation at 1500 x *g* for 10 min and cfDNA was extracted using Qiagen's Circulating Nucleic Acids kit as previously described [6]. Libraries were prepared and sequenced at DFCI using a pre-production ctDx-Lung kit (Resolution Bioscience, Redmond, WA) spanning exons and some introns of 20 NSCLC-associated genes (*AKT1*, *ALK*, *BRAF*, *CD274*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR3*, *JAK2*, *KRAS*, *MET*, *MYC*, *NRAS*, *NTRK1*, *PIK3CA*, *PTEN*, *RET*, *RICTOR*, *ROS1*, and *TP53*) [6]. Importantly, libraries are hybridized with 40 nt targeting probes with each probe possessing an additional tail sequence that is complementary to a biotinylated pull-down oligonucleotide. Following hybridization and purification of probe-clone complexes, primer extension of the probe is then used to copy the genomic sequence and tag information, allowing for high on-target rates as well as the association of each read with the identity of its cognate probe. Sequencing was performed on an Illumina NextSeq500 followed by cloud-based, bioinformatic variant calling. Copy number variations were not address in this study as the challenges of quantifying these variants in cfDNA were outside the scope of this study. Median depth of coverage across samples was 2300 × . Personnel responsible for performing specimen preparation, sequencing, and variant calling were blinded to the known tumor genotype and the G360 results. Plasma from two additional fusion negative NSCLC patients were included as negative controls. Once plasma NGS results were locked, laboratories were unblinded for additional post-hoc analyses. Both companies were given the opportunity to bioinformatically re-review cases after being informed that fusions were present.

3. Results

Our cohort of 16 patients with advanced NSCLC were positive by diagnostic tumor genotyping in our institutional pathology laboratory for an *ALK* (11), *ROS1* (2), or *RET* (3) rearrangement and had undergone clinically-ordered G360 testing. All were treated with a matched TKI, with 10 (63%) having prolonged time on therapy exceeding 11 months and 5 additional cases with shorter benefit but having at least a minor response to therapy (Supplemental Table); only one patient was refractory to treatment, a patient with a RET-fusion treated with alectinib. Clinically-ordered G360 testing detected the fusion in 7 cases (44%) while ctDx-Lung detected the fusion in 13 cases (81%). 6 cases were detected by ctDx-Lung and missed by G360 (Fig. 1); in 4 of these discordant cases G360 detected the same *TP53* mutation as the ctDx-Lung at a similar allelic fraction (AF) as the ctDx-Lung results, and in 3 cases the paired plasma was collected within 1 week. Note that in 2 of these cases, the detected *TP53* mutation was confirmed to be present on tumor NGS, confirming the presence of circulating tumor DNA despite lack of fusion detection. In the 7 fusion cases detected by both assays, ctDx-Lung consistently reported a higher AF% then G360 (median difference 7.0%), possibly indicating more efficient capture of cfDNA fragments corresponding to fusion breakpoints. In the remaining 3 cases, neither the fusion nor mutations in tumor suppressors, such as *TP53*, were detected (see Supplemental Table 1) by either assay suggesting that these false negatives may be due to low tumor DNA content. Neither assay reported fusions in the 2 negative control cases.

ctDx-Lung uses tiled, directional hybrid-capture probes and subsequent primer extension to form a sequencable clone of the breakpoint and gene fusion partner (Fig. 2). 10 detected fusions in this cohort included previously well-documented events such as EML4-ALK, KIF5B-RET, and CD74-ROS1, while 3 rare fusions were also detected (ADAM17-ALK, ALK-ALK, and EML4-RET) (Supplemental Table 1). The ADAM17-ALK, ALK-ALK, and two EML4-ALK cases were predicted to be out-of-frame or inverted, though all but one of these cases (one EML4-ALK) showed clinical benefit to TKI. These results are consistent with a previous report showing that DNA-based breakpoint calling is an unreliable predictor of the resulting transcript [7]. Notably, in one case where the ctDx-lung kit identified a rare, inverted fusion partner (ADAM17-ALK), G360 reported a reversed ALK-EML4 fusion.

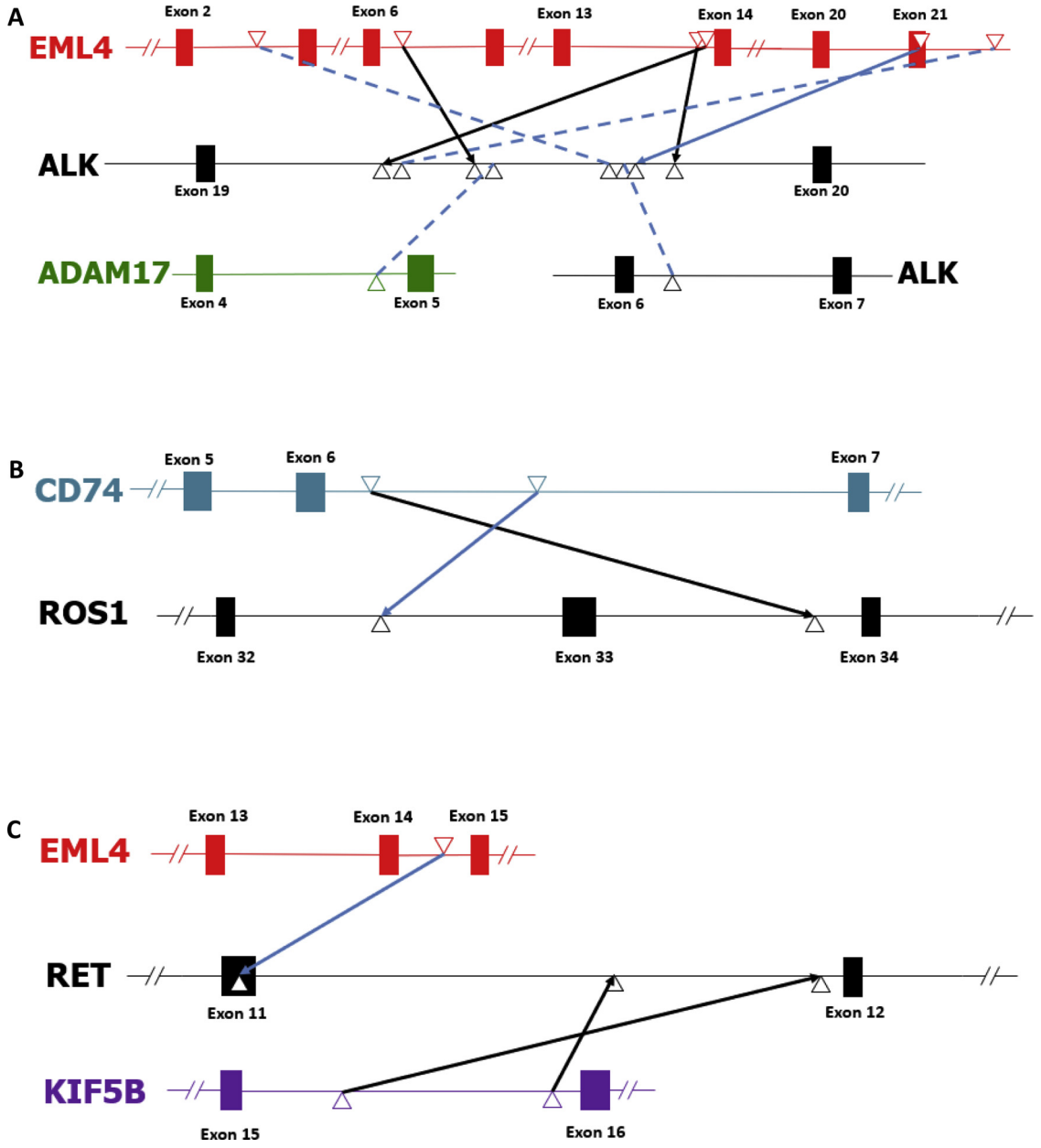

Fig. 2. Schematic of the 13 gene fusions detected in cell-free DNA (cfDNA) using the ctDx-Lung kit. For each oncogene (**A**, *ALK*; **B**, *ROS1*; **C**, *RET*), the oncogene is shown in black and the fusion partners in color. Blue lines represent fusions not detected by Guardant360. Solid arrows represent in-frame rearrangements, and dotted lines represent inverted/out-of-frame rearrangements.

4. Discussion

Unlike previous concordance studies [3], here we focus on a subset of advanced NSCLC with known tumor genotypes of interest and, through collection of plasma in conjunction with clinically ordered plasma NGS, have identified unappreciated limitations in detecting targetable fusions in cfDNA. Importantly, we use tumor genotyping as a reference standard, allowing examination of the role of technical differences in the performance of these two plasma NGS assays. We note that some patients in our cohort were ALK positive on FISH only, which has a somewhat higher false-positive rate than NGS [8], but all three patients in our cohort who had only tumor FISH testing also responded to TKIs, suggesting that these were indeed true positives.

Low tumor DNA shed does not fully explain the impaired sensitivity seen in our study – differences in fusion detection were noted between the two plasma NGS assays despite both assays detecting the same TP53 mutations in several samples. Numerous prior reports of plasma genotyping efforts using G360 have described an incidence of gene fusions lower than expected for NSCLC [1,5], which may be related to the impaired sensitivity we observed in this study. Additionally, studies of ALK resistance using G360 have detected ALK resistance mutations without detecting the ALK fusion [9], suggesting impaired sensitivity specifically for the fusion and not for ALK point mutations.

While reported AFs are less important in clinical practice [1] than the presence or absence of a given mutation, the consistently lower AFs of fusions reported by G360 compared to ctDx-Lung (even when common SNVs are of similar AF) suggests that G360 may have a specific technical limitation with regards to gene fusions. We hypothesize that differences in the design of hybrid-capture based NGS assays may explain the variable sensitivity we identified [10]. Capture probe design is especially important in fusion detection due to the variability of breakpoint locations and the fragmented (~150bp) nature of cfDNA.

One technical characteristic of ctDx-Lung is its use of short (˜40 nt) capture probes rather than more standard 120 nt probes, which may offer more efficient hybridization to cfDNA fragments [6]. Another technical difference is the use of primer extension, which copies the sequence of the fusion partner regardless of the identity of the partner. Rare, inverted fusion events were overrepresented in cases detected by the ctDx-Lung alone (3/6), while G360 detected one rare variant, though the reported variant (ALK-EML4) differed from what was reported with the ctDx-Lung (ALK-ALK).

Although the primer extension step employed in the ctDx-Lung protocol likely enhances rare variant calling, bioinformatic filtering of these inverted sequences cannot be ruled out as a potential source of false negative calls by G360. When contacted for blinded re-review, G360 was able to apply the latest version of their bioinformatic pipeline to sequencing data from 14 of 16 cases, confirming all fusions previously reported by G360 and detecting fusions in two additional cases. In one case, re-review by Guardant identified a common EML4-ALK fusion which had been present in tissue, but again at a lower AF% than reported by ctDx-Lung (1.4% vs 8.0%, respectively). In the other case, manual re-review by both Guardant and Resolution Bioscience identified low level evidence of an EML4-ALK fusion (0.1% AF). Together these findings suggest that effective bioinformatics, which are constantly evolving, are needed to properly call variants. Similar to previous studies,[11] most discordant calls between plasma assays are at low AF% as these are especially susceptible to stochastic differences.

Our study has clear limitations, most notably the small sample size. Actionable fusions, while a good model for evaluating complex variants, are rare in NSCLC, and routine clinical plasma NGS is relatively new, thus precluding a larger sample size in this retrospective analysis. Additionally, while samples were batched together for sequencing with ctDx-Lung, G360 testing has been used on patients variably over the course of two years. In this time, the G360 assay has evolved through four commercial iterations (v14.0- v17.0). Follow-up comparison studies should try to use latest version of all assays. Finally, the two plasma specimens studied for each patient were not routinely collected simultaneously. Of the 6 fusion cases reported by ctDx-Lung and missed by G360, 3 had plasma collected within 1 week. Of the other 3 cases, 2 reported the same *TP53* SNV. In the remaining case, G360 failed to report the EML4-ALK fusion but did report SNVs in *TP53*, *ALK*, and *SMAD4* as reflected in tissue (ctDx-Lung analyzed plasma from 6 months later and reported only EML4-ALK and a low-AF SNV in *NRAS*). Thus, differences in collection time do not adequately explain discrepancies in fusion reporting for this cohort.

5. Conclusions

In conclusion, detection of gene fusions by plasma NGS is more variable than has been observed for point mutations and indels. While our study was limited in sample size, ad hoc analysis, and the use of multiple versions of G360, our results are nonetheless thought-provoking and suggest that technical differences between two apparently similar plasma NGS assays may be an underappreciated source of discordance. Future studies should focus on orthogonal benchmarking using tumor RNA to better characterize the presence of heterogeneous fusions reported here and to confirm the clinical utility of detecting these genomic variants in plasma. NGS of plasma cfDNA clearly promises to offer increased access to precision therapies for patients making it important to adequately understand the performance characteristics and interpretations of these assays. Our data suggest continued efforts are needed to improve existing assays to fully leverage their potential to impact patient care.

Conflicts of interest

Lee P. Lim and Kristy T. Potts are employees of Resolution Bioscience and have stock-based incentive. Lynette M. Sholl is a consultant for Foghorn Therapeutics and AstraZeneca and is on the scientific advisory board of LOXO Oncology. Geoffrey R. Oxnard has received consulting fees from Inivata, AstraZeneca, Sysmex, DropWorks, GRAIL, Takeda, and Loxo Oncology and has received honorarium from Guardant Health and Foundation Medicine. Cloud P. Paweletz has received honoraria from Bio-Rad and AstraZeneca, is a co-founder of XSphera Biosciences, is on the scientific advisory board of DropWorks and XSphera Biosciences, and has received research funding from Guardant Health. All remaining authors have declared no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.06.004.

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