Editorial

RELAY phase 3 randomized study: a step closer to the clinic for ctDNA in non-small cell lung cancer treatment monitoring?

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Liquid biopsy is gaining traction as a non-invasive strategy for molecular characterization, screening, tumour genotyping, prognosis, monitoring of treatment response and disease progression, detection of resistance development and relapse in non-small cell lung cancer (NSCLC). Application in routine clinical practice is largely limited to resistance detection. Significant hurdles remain for routine clinical implementation of other emerging indications. These hurdles include choice of circulating tumor DNA (ctDNA) biomarker features, establishing clinical utility, standardization of clinical laboratory methods, establishing landmark and/or surveillance points, cut-offs for biomarker levels and clinical treatment decision strategy. A meta-analysis of 51 early-stage NSCLC liquid biopsy studies while highlighting commonalities, demonstrated significant heterogeneity between studies due to variability in liquid biopsy biomarkers, detection technologies, lack of a standardized ctDNA detection manual, etc. (1). The American Society of Clinical Oncologists and the College of American Pathologists acknowledge conflicting evidence for clinical utility and validity since many investigations were conducted outside of clinical trials (2). The rigor of a clinical trial setting is necessary to determine appropriate biomarker features, establish clinical validity and utility, and patient safety.

In this exploratory, proof-of-concept liquid biopsy addendum study, Nishio *et al.* take a stab at some of these issues (3). They present an addendum to the RELAY phase 3 randomized double-blinded, placebo-controlled study of the impact of ramucirumab (a human immunoglobulin G1 vascular endothelial growth factor receptor 2 antagonist) plus erlotinib (RAM + ERL) on cell-free DNA (cfDNA) from patients with untreated metastatic NSCLC with EGFR activating mutations. The initial RELAY study found that in comparison with erlotinib and placebo, RAM + ERL had improved progression-free survival (PFS). The addendum study examined the connection between liquid biopsy biomarkers and treatment outcomes in the context of the RELAY clinical trial. Exploratory endpoints of the addendum study were co-occurring gene alterations at baseline and impact on treatment outcomes, and epidermal growth factor receptor tyrosine kinase inhibitor (EGFR TKI) resistance mechanisms, T790M mutation rates post-study treatment discontinuation, and changes in cfDNA concentration and cfDNA fragment size through treatment.

The specific objectives were to elucidate combination drug interactions with respect to resistant mutation occurrence, and the relationship between biomarkers and treatment outcomes. Other highlights include time points for liquid biopsy-informed therapeutic decision-making; the utility of different laboratory approaches, sensitivity and specificity of biomarker prognostication. To address these objectives, plasma samples were collected at multiple cycles for ctDNA analysis. The diagnostic techniques employed were real-time PCR, droplet digital PCR (ddPCR), amplicon-based next generation sequencing (NGS) and

Table 1 Summary of biomarker features and correlation with clinical outcome

Biomarker features	Method	Correlation with clinical outcome	Concordance with other studies
cfDNA concentration	Real-time PCR	Yes	New finding (4)
ctDNA mutation allelic fraction	ddPCR, NGS	No	Conflicting results (5,6)
DNA fragment size	Bioanalyzer	Yes, and new proposed mechanism of action	Yes (4)
Co-occurring mutations	NGS	No	No (7,8)
ctDNA T790M mutation clearance from baseline at cycle 4	NGS, ddPCR	Yes	Yes (9)

cfDNA, cell-free DNA; ctDNA, circulating tumour DNA; ddPCR, droplet digital PCR; NGS, next generation sequencing.

electrophoretic mobility analysis. cfDNA features included cfDNA concentration, cfDNA fragment size, mutant allele count, and co-occurring gene alterations. These objectives reflect roadblocks to routine clinical use of liquid biopsy in anti-EGFR TKI therapy monitoring in NSCLC patients.

In using four methodologies with varying complexities, Nishio *et al.* underscore the question of appropriateness of methodology (*Table 1*).

ctDNA studies have utilized methodologies ranging from real-time PCR, digital PCR, e.g., droplet digital PCR, beads emulsion amplification magnetics (BEAMings), to NGS. The least complex, real-time PCR, has the advantage of being inexpensive, and relatively easy to use and analyze. However, its limitation is that only single target can be interrogated. ddPCR utilizes a partition-based technique with a Poisson distribution model to quantify the presence of rare variants. It is capable of multiplexing up to 6 targets. It has the advantage of absolute quantification and exquisite sensitivity. Its ease of use and low cost is showcased by the greater multiplicity of time-points for ddPCR (baseline, cycle 4, cycle 13 and every 6 cycles thereafter) as opposed to just 3-time points for NGS in this study by Nishio *et al.* They report that the limit of detection (LOD) of ddPCR appears to be superior to the NGS technique used, with ddPCR having higher detection rates for ctDNA mutations compared to NGS. This is indicative of the problem of the variability that occurs from using different techniques even within the same study. Despite the comparatively limited LOD of NGS in this study, it should be noted that new NGS techniques e.g., unique molecular indices (UMIs) with error-correction have significantly improved the LOD of NGS mutation detection. In addition, NGS is able to interrogate a wider range of targets. In this study, a 22-gene NGS panel was used compared to the single target for ddPCR. However, NGS assay cost and complexity of testing and analysis may be a drawback in broad clinical adoption for serial monitoring compared to ddPCR.

ctDNA biomarker features remain an intensive area of investigation. Emerging data show how different features could be used to predict patient outcomes and tumour biology. These biomarker features reflect the cellular process of ctDNA generation. The degree of tumour shedding of ctDNA into the extracellular space determines the presence and the level of ctDNA and is determined by factors such as tumour volume and tissue type. Nonshedders do not have detectable ctDNA and are a cause of the moderate sensitivity for ctDNA analysis. Within ctDNA shedders, cfDNA concentration has been shown to impact PFS. In a retrospective study of patients with advanced NSCLC, Peng *et al.* report an inverse relationship between cfDNA concentration and PFS (10). Lower baseline cfDNA concentration showed an increase in PFS. cfDNA before the third treatment was also an independent factor for disease progression. In the current study, Nishio *et al.* evaluate multiple biomarker features including cfDNA concentration, ctDNA mutations, ctDNA mutation allelic frequency, ctDNA size profile. As with similar studies, they exclude non-shedders from analysis. They report that patients with EGFR mutation clearance at cycle 4 had improved PFS. This is concordant with previous findings that ctDNA mutation detection as well as mutation type, at baseline and its clearance with TKI treatment is an independent prognostic factor for progression free survival and overall survival (9).

Surveillance analysis of ctDNA allelic fraction has been evaluated by longitudinal mutation tracking for tumour progression. High allelic fraction may be reflective of tumour burden. Allelic fraction at baseline has been reported as a poor prognostic indicator (5). However, Gray *et al.* were unable to establish a correlation between allelic

fraction and PFS in their analysis (9). Nishio *et al.* also find that allelic fraction, when partitioned as high *vs.* low, did not impact PFS in their study population.

Targeted detection of T790M mutation is limited by a relatively lower sensitivity 60% to 70% despite high specificity (80–98%) (11). The relatively low sensitivity reflects the fraction of non-shedding tumours, and the assay detection limits such that negative results may actually be due to ctDNA below detection threshold. Abbosh *et al.* showed that tumor volume <10 cm was frequently associated with non-shedding (6). A number of approaches attempt to improve on sensitivity. Detection of ctDNA can be classified into tumour-informed and tumour-uninformed/tumour-agnostic approaches. The tumour-informed approach involves baseline sequencing of patient tumour tissue to identify mutations, which are then used to design assays for subsequent monitoring of ctDNA for each specific patient. For instance, Abbosh *et al.* perform baseline whole exome sequencing to identify variants which were used to design patient specific panels using an anchor multiplex PCR for subsequent NGS sequencing on patient ctDNA (6). The tumour-uninformed approach does not require prior knowledge of mutations in a patient's tumour. While many tumour-uninformed NGS approaches suffer from lower sensitivity, new innovative approaches, e.g., Cancer Personalized Profiling by Deep Sequencing (CAPP-Seq) increase sensitivity by interrogating a broad range of recurrently mutated intronic and exonic targets in cancer, with reported ctDNA detection in 100% of patients with stage II–IV NSCLC and 50% in stage I and a specificity of 96% for allele fraction down to 0.02% (12). Nishio *et al.* employ a tumour-uninformed approach with the commercial Ion AmpliSeq Colon and Lung cancer panel v2 comprising of 22 pre-selected cancer-related genes. NGS could be used to interrogate for co-occurring prognostic and resistant mutations; or to improve sensitivity for ctDNA detection. Nishio et al utilize NGS for the purpose of detecting co-occurring mutations. The treatment-emergent mutations detected include EGFR, FGFR3, KRAS and TP53. Some of these have prognostic and secondary resistance implications. For instance, baseline and emergent TP53 mutations have been reported as a poor prognostic indicator and have been associated with small cell transformation (8). Nishio *et al.* however do not find any prognostic or predictive association for TP53.

ctDNA is released into the extracellular space either by passive or active processes. Passive release occurs after tumour apoptosis or necrosis, resulting from hypoxic or metabolic stress encountered during rapid tumour proliferation (13-16). Necrosis typically produces longer ctDNA fragments. cfDNA from apoptosis has a characteristic ladder-like profile in normal and cancer patients. cfDNA size profile possesses a modal size peak of 167 bp, reflective of the length of DNA spiraling around a nucleosome and the attached linker DNA. The ladder consists of successive peaks 10 bp apart, arising from 143 bp then diminishing in length. This profile is related to tissue type, cancer type and stage. It also has been suggested to correlate with clinical outcome (17,18). The analysis of ctDNA profile, also known as cfDNA fragmentomics, is a novel approach to ctDNA analysis (19,20). ctDNA fragments (cancer derived cfDNA) are shorter than non-cancer cfDNA. In addition, these shorter ctDNA fragments are the predominant source of mutant alleles, implying that size analysis may be a useful approach in differentiating fragments arising from tumour DNA from non-tumour DNA, as occurs from clonal hematopoesis (21,22). Furthermore, the end sequences of ctDNA fragments are nonrandom, frequently possessing "preferred end sites" that may be used to identify cancer-derived ctDNA (23,24). Wang *et al.* in a study of NSCLC patients who underwent curative-intent surgical resection showed that a cfDNA fragmentomics-based model using whole genome sequencing had superior sensitivity and recurrence predictive power compared to traditional targeted sequencing for ctDNA detection (4). Studies in ctDNA size analysis have utilized methods such as whole genome NGS, electrophoresis, PCR, etc. In this study, Nishio *et al.* use an electrophoretic mobility assay, a simple and cost-effective assay capable of clinical implementation on a large scale. They detected a negative correlation between change in cfDNA concentration and change in cfDNA fragment size with increased shorter fragment size for the RAM + ERL arm.

As ctDNA represents a snap-shot of the complete tumour genome at a point in time due to its short plasma half-life, longitudinal evaluation of its fractional components could give an understanding of clonal evolution with treatment. Various studies have demonstrated the use of liquid biopsy in monitoring clonal evolution of tumour in response to therapy. In a preliminary study of EGFR mutated NSCLC patients on third line TKI, osimertinib, Fuchs *et al.* report the detection of various mutations at disease progression and the impact of these mutations on disease features and patient outcome. TP53 was the most commonly detected mutation at progression, followed by MET amplification (7). Other resistant mutations included KRAS, RB1, BRAF, MYC

amplification. The detected mutation types were used to inform alterations in treatment strategies, e.g., combination of MET inhibitor with osimertinib was done in patients with secondary MET amplification. Other changes in combination therapeutic strategies included osimertinib with chemotherapy, osimertinib with immunotherapy, osimertinib with 1st generation TKIs, 1st generation TKIs with MEK targeted therapy etc. The study also correlated clinical behaviour with mutation profiling, demonstrating that tumours with RB1, TP53 and PIK3CA were at highest risk for small cell lung cancer transformation, a finding that was concordant with other studies (8). Abbosh *et al.*, investigating patients with early-stage NSCLC over a 3–5-year interval, tracked a median of 64 subclonal mutations to decipher subclonal evolution at relapse (6). They demonstrated that primary tumour subclones with larger preoperative cancer cell fraction (CCF) were most frequently persistent postoperatively, were associated with greater relapse expansion and metastatic potential relative to those with lower CCF. ctDNA mutations was used to determine upregulated pathways and other features of tumour biology, e.g., proliferation rates. They showed that in up to 43% of studied tumours, a subclone had progressed to total tumour replacement at all tumour sites at recurrence. Thus, estimating and profiling subclonal expansion by ctDNA preoperatively may permit prediction of metastatic subclones and enable early intervention strategies.

Nishio *et al.* report treatment emergent gene mutations in EGFR (T790M and 870R), PTEN, FGFR3, KRAS and TP53. Superior PFS was seen in the liquid biopsy of patients with undetectable EGFR activating mutations at cycle 4 compared with detectable. This is in keeping with the AURA and FLAURA studies (9). For the RAM + ERL cohort, total cfDNA increased with treatment but the increased cfDNA species had shorter fragment size compared with controls. The authors implicate increased tumour cell apoptosis as the cause. Due to the fact that longitudinal kinetic profile cfDNA with shorter fragment size was inversely correlated with EGFR mutation clearance in the RAM + ERL group, the authors proposed a mechanism of action of therapeutic killing by continuous tumour cell apoptosis on non-EGFR mutated cells, suggesting that RAM enhanced the anti-tumour effect of ERL. This interesting finding and their suggested explanation have interesting implications for liquid biopsy therapeutic monitoring. This finding taken together with the Abbosh *et al.* study of subclonal evolution, suggest potential utility of liquid biopsy in dissecting clonal subpopulation to understand individual subclonal tumour biology, clonal evolution and the responsiveness of each subclone to specific agents in combination therapy. This evaluation could potentially be used to optimize drug combination of the ongoing treatment regimen.

In conclusion, Nishio *et al.* have presented a study with interesting findings and new implications for liquid biopsy in NSCLC. Considering the range of complexities of liquid biopsy testing modalities, all four molecular techniques were complementary. Importantly, the study employed a simple electrophoretic mobility assay, bioanalyzer, for its fragmentomics study. Despite its simplicity, it offers significant information for potential clinical decision-making. Nishio *et al.* show that landmark and surveillance analysis were important and complementary for prognostication. This study expands interesting areas for further investigation. For instance, is it possible to differentiate between ctDNA shed from cells killed by therapy or actively secreted ctDNA from treatment resistant cells (25). This has implications for understanding subclonal biology and treatment responsiveness. Given the multiplicity of EGFR TKIs, can the findings of a specific therapy, e.g., RAM + ERL be transposed to other therapy? Preliminary findings from some studies with different treatment lines suggest that EGFR mutation clearance is not specific for the treatment type. How can ctDNA mutations be differentiated from clonal hematopoesis? Paired mutational analysis of ctDNA with peripheral blood cells has been suggested. Fragmentomics also offers a solution. The question of standardization remains unresolved particularly in quantitative monitoring, and fragment size profiling. As assay sensitivity increases, standardization of allelic fractions cut-offs would be required for cross-study comparison. Finally, this study complements others that provide preliminary pointers that liquid biopsy could further expand the horizon of precision medicine by tracking treatment mechanisms of action and subclonal tumour biology in order to optimize combination therapy in real time.

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