



Liquid biopsies in non-small cell lung cancer management: what can we learn from methylation status and mutant allele frequencies?

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In the age of precision oncology, the field of targeted therapies for non-small cell lung cancer (NSCLC) has rapidly grown, altering the treatment dynamics for thoracic physicians. In addition to the traditional imaging methods of monitoring lung disease, genetic analysis of a patient's tumors is a guiding factor in this process. Genetic interrogation is especially important in cancer types represented by a high percentage of patients with known actionable driver alterations. This scenario is exemplified in epidermal growth factor receptor (EGFR)-mutant lung adenocarcinoma, where the best choice of agent amongst a crowded field, depends on the major mutant representative of the heterogeneous tumor population. Treating mutant EGFR with tyrosine kinase inhibitors (TKI) in patients with advanced NSCLC has been one of the biggest success stories in targeted cancer therapy. Unfortunately, the therapy eventually fails for all patients, and the disease progresses. Targeted EGFR-tyrosine kinase inhibitors offer selective pressures which constantly alter the dynamics of the tumor cell population, leading to frequent clonal expansions of the most fit clones (1,2). To this end, resistance mutations or mechanisms after prolonged treatment with EGFR TKIs, such as the current first-line osimertinib, are being reported at an increasing rate, where

each TKI has a unique spectrum of resistance mechanisms that arise (3-5). Biopsy of lung adenocarcinoma is a complicated procedure which often requires sedation, and only provides a spatial and temporal snapshot of the isolated region at the time of collection. Ideally, biopsies would be collected when making all treatment decisions, following any indication of disease progression (PD), as sensitivity to treatment does depend on mutational status (6). In the case of lung adenocarcinoma, this is not always feasible, as repeated biopsies subject the patient to unnecessary pain and complications.

The field has come to realize an alternative to our traditional view of a biopsy, as the evidence for the importance and reliability of circulating tumor DNA (ctDNA) in NSCLC has steadily grown (2,7-10). Free DNA is cleared from the bloodstream within ~30 minutes—lending itself the ability to truly provide a real-time snapshot of the genetic alterations predominating at the time of a minimally invasive blood draw or urine collection, a term coined “liquid biopsy” (11-13). These DNA fragments are derived from tumor sites, both primary and metastatic, as cells are subjected to necrosis and apoptosis, unbiasedly shedding their tumor DNA into the bloodstream. The random nature of this release method

Table 1 Summary of the molecular patterns identified from plasma ctDNA

Group number	maxAF & methylation ratio patterns
1 (n=3)	maxAF and methylation ratio correlated tightly with one another. There was a decrease in both values following the start of osimertinib treatment, and a steady increase in both values upon radiological PD. All patients developed C797S, with an average molecular progression detected at 2.8 months prior to radiological progression
2 (n=2)	Methylation ratio saw a sharp increase following osimertinib initiation, followed by a decrease during partial response. Methylation remained low throughout efficacious treatment, until a slight increase was demonstrated 2 months prior to radiological disease progression
3 (n=1)	maxAF gradually increased over time, coinciding with radiological disease progression. Methylation status remained unchanged
4 (n=2)	Methylation fluctuated throughout treatment, offering no predictive power, perhaps due to the relatively low amounts of ctDNA detected in these patient's samples

allows for presentation of both intra- and inter-tumoral heterogeneity, features often missed in the traditional mode of lung biopsy (14). Xia *et al.* contributed to this body of literature with their 2020 article in this edition of Translational Lung Cancer Research, in which they utilized serial blood draws to assess the prediction power of disease progression by somatic mutation and methylation status of select genes from ctDNA (15).

Briefly, Xia *et al.* identified 8 patients who were treated with osimertinib for EGFR-T790M-positive advanced metastatic lung adenocarcinoma as part of the AURA17 phase II trial (NCT02442349). All patients had received at least one prior treatment, ranging from first generation TKIs, erlotinib or gefitinib, platinum-based chemotherapy, or palliative radiotherapy. After collecting baseline blood samples prior to beginning osimertinib treatment, each patient received 80 mg of osimertinib daily. Blood samples were collected longitudinally during the treatment course, until clinical symptoms and radiological modalities demonstrated disease progression. The authors used the clinically accepted Response Evaluation Criteria in Solid Tumors (RECIST version 1.1) definition for disease progression (16). Blood samples were used to assess paired somatic mutation by capture-based targeted DNA sequencing and DNA methylation status, by bisulfite sequencing.

Somatic mutation status of ctDNA was reported as maximum allele fraction (maxAF), defined as the highest fraction of mutant allele detected, regardless of the gene or mutation site. As this number changed over time, maxAF was normalized to the maximum value detected over the study, to describe the fraction of ctDNA in circulation at a specific time for each patient. It should be noted that for 7 of the 8 patients, an *EGFR*-sensitizing mutation was

detected as the maxAF (ranging from 4.00% to 6.29%), with the outlier having a *TP53* at maxAF of 4.95%.

Methylation levels were reported as methylation ratios (MR), calculated by dividing the number of positive differential methylation blocks by the total differential methylation blocks within the sample. The authors demonstrated the sensitivity of their methylation detection system using commercially available CpG methylated genomic DNA, showing better detection with higher MRs. Despite this, their system was capable of detecting methylated DNA down to an MR of 0.0001. When the correlation between MR and maxAF was calculated, the analysis revealed a positive correlation between the two values in 85 plasma samples, especially when samples were separated by patients ($P=0.0002$). Further, those plasma samples that had detectable somatic mutations, had significantly higher methylation levels than those with no detectable mutations ($P=0.0003$), or the samples of healthy controls ($P=0.0018$). The MR and maxAF values were incorporated using the described equation, to create an MR disease progression prediction model, or MR model score.

Pairing of the longitudinal analyses of MR and maxAF values with radiological imaging and treatment response clustered patients into four general groups (*Table 1*). Group 1 was defined by patients who first displayed a significant reduction in maxAF and methylation levels following the start of osimertinib treatment. Throughout treatment they remained low, until a gradual increase in both values was observed, with methylation level eventually reaching a similar MR detected at baseline when radiology showed PD confirmation. This group, which consisted of three patients, all developed the classical osimertinib resistance mutation, *EGFR* C797S, with an average detection time of this mutation 2.8 months prior to radiologic disease

progression.

Group 2 consisted of two patients who had a sharp increase of MR following osimertinib initiation. Their MR then decreased when the patients both displayed a partial response to the treatment, defined as their best response during the study. These levels remained low throughout the duration of treatment, until 2 months prior to disease progression, at which time MR showed only a slight increase.

Group 3 was represented by a lone patient, who showed an unchanged methylation level throughout treatment and disease progression, offering little predictive power. However, maxAF gradually increased, until it reached double the baseline maxAF, which coincided with the patient's disease progression. The last group of patients, Group 4, consisted of two patients whose methylation status fluctuated without pattern throughout treatment. The authors suggest that this may be due to the relatively low amounts of ctDNA that could be detected throughout the study in these patient's samples. This group reflects the intrinsic interpatient variability of ctDNA levels in lung adenocarcinoma, surely a challenge of applying this technology in the clinic.

Molecular disease progression, defined by the authors as "the emergence of new mutations or significant increases in allele fraction (AF) of existing driver mutations", was observed in 5 of the 8 patients prior to the detection of radiological progression. Perhaps most exciting, this molecular PD, on average, was detected 1.9 months prior to radiological PD, and as early as 2.7 months in 2 of the 5 patients. Further analysis of these trends in MR and maxAF revealed that treatment efficacy was reflected molecularly by a significant reduction in both methylation status and maxAF, where the converse, a significant increase in either value, reflects an imminent physical disease progression.

This work by Xia *et al.* contributes additional evidence to the growing camp of researchers demonstrating the utility of tracking the therapeutic response of patients through ctDNA isolated from a noninvasive blood draw (17,18). Though their study is limited by a small sample size, they are not alone in observing a strong correlation between TKI response and the presence or level of mutant EGFR sequences in plasma or urine (2,8,9,19). A 2017 study of 45 lung adenocarcinoma patients with confirmed EGFR driver mutations revealed that the levels of plasma mutant-EGFR correlated with treatment efficacy (9). Here, a significant reduction was seen during the first two months of TKI treatment, and

a significant increase correlated with changes in tumor diameter or the development of new lesions determined via radiographic imaging. Moreover, they were able to detect the *EGFR* T790M resistance mutation as early as 5 months prior to radiological PD in some patients (9).

Xia *et al.*'s paper also highlights the growing trend of researchers who are taking full advantage of the spectrum of information that can be extracted from a liquid biopsy. Besides the genetic information mentioned above, epigenetic information, like methylation status, has been correlated with patterns of cancer prognosis and treatment response—a trend seen in a multiple cancer types (20,21). Although believed to be unique in each cancer type, in general, tumor cells are host to global hypomethylation, and regional hypermethylation at CpG-rich promoters of tumor suppressor genes, collectively leading to increased genomic instability, activation of proto-oncogenes, and inactivation of tumor suppressor genes (22).

The *SEPT9* gene encodes the protein Septin 9, a complex GTP-binding protein, broadly classified as a tumor suppressor, which is involved in cell motility, proliferation, actin and microtubule dynamics, angiogenesis and exocytosis, to name a few functions. Hypermethylation of CpG island 3 in the promoter of the *SEPT9_v2* transcript, subsequently resulting in gene silencing, was shown to tightly correlate with colorectal cancer (CRC) development. Further studies proved this epigenetic mark could be used as a biomarker for CRC, which could be detected through high-sensitivity real-time PCR of patient's blood samples. In May of 2016, these findings translated to the first and only commercially available FDA-approved blood test for ctDNA methylation (Specifically methylation of *SEPT9*; m*SEPT9*) used for cancer screening, Epi proColon[®]. With a sensitivity of 48% and specificity for CRC of 92%, this test is not a replacement for the gold standard in CRC detection, a colonoscopy, but rather can be used in the population of patients who refuse colonoscopies for one of many reasons (23,24).

But our capabilities of detecting altered methylation patterns from cell-free DNA are steadily improving. A large study in colorectal cancer by Luo *et al.*, published in January of 2020, identified a panel of genes which could be used as a CRC-specific methylation signature. They applied a machine learning program to train on 801 CRC patient samples, and 1,021 healthy patient samples of cell-free DNA. Their algorithm was capable of discerning CRC *vs.* non-CRC patients, and effectively predicting patient prognosis by methylation signature. By the end of the

analysis, they identified a single methylation mark which had 89.7% sensitivity and 86.8% specificity for detecting both cancerous and precancerous colon lesions (25).

This study by Luo *et al.* has several strengths that are lacking from the present study, but nonetheless, their paper fortifies the arguments made by Xia *et al.*, while demonstrating the feasibility of expanding their project. Adaptations of the larger scale workflow from Luo's group could be utilized to set up a similar unbiased analysis across a large treatment cohort of lung adenocarcinoma patients in order to examine lung-specific ctDNA methylation patterns. This work also highlights the obstacles that we are yet to overcome in translating this technology to the clinic. The standardization of blood collection time points with respect to treatment, sequencing and methylation analyses, and the improvement of sensitivity and specificity, would all be required to perform this prospective study on a large scale, or in a clinical setting. The ultimate goal, of course, would be to integrate liquid biopsies into NSCLC treatment regimens, with the hope of detecting a molecular progression earlier than that of a physical progression, allowing physicians to make more acute treatment decisions.

In the age of targeted therapy in lung cancer treatment, molecular dynamics of a disease are more important to treatment decisions than ever before. Despite the shortcomings of this work, Xia *et al.* and numerous other groups have demonstrated how ctDNA sequencing technology is capable of gleaning more than just mutational status of select genes. Epigenetic information can be extracted, and this work showcases its utility in predicting treatment responses in lung cancer. It may be seen that this technology is most useful in cancer types which do not image well, and are distally located, making repeat biopsies throughout treatment difficult. Nonetheless, liquid biopsies seem to be carrying the field into the future of cancer management.

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Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tlcr.2020.04.05>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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