Editorial Commentary

Circulating tumor DNA (ctDNA)—the next generation biomarker in non-small cell lung cancer patients treated with immunotherapy?

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Lung cancer accounts for the most cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) represents around 80% of lung cancer cases with adenocarcinoma being the most prevalent subtype (1). For lung adenocarcinoma of advanced or metastatic disease, and without activating genetic aberrations in *EGFR*, *ALK*, *ROS1*, *BRAF*, *MET*, *RET*, and *NTRK*, systemic therapy with immune-checkpoint inhibitors (ICIs) has become the first-line treatment choice, representing the standard-of-care for the majority of lung adenocarcinoma patients with advanced or metastatic disease. Depending on the status of programmed cell death 1 ligand 1 (PD-L1) expression, ICIs may be administered alone, as monotherapy (PD-L1 expression >50%), or in combination with platinum-based chemotherapy (PD-L1 expression <50%). Despite improved clinical outcomes for lung cancer patients receiving ICIs compared to chemotherapy alone, resistance is inevitable and calls for improved biomarkers to predict clinical response (2). Although several biomarkers have been evaluated for lung cancer patients receiving ICIs, including tumor mutational burden, microsatellite instability and gene-expression profiles, there is lack of consistency in these biomarkers to predict clinical outcome (3-5).

In a recent publication, Murray *et al.* investigated the impact of monitoring plasma-derived circulating tumor DNA (ctDNA) longitudinally in 30 NSCLC patients (6). The criteria for inclusion in the study was at least one cycle of ICI or a combination of ICI and chemotherapy, at least two plasma samples for ctDNA sequencing, at least one buffy-coat sample for white blood cell (WBC) whole exome sequencing (WES) and/or WES of tumor tissue. This was accompanied with imaging until a minimum of 6 months after treatment initiation. Out of 30 patients, 29 patients had matched WBC DNA while 17 patients had matched WES for both tumor and WBC DNA. Uncovered variants in plasma were cross-referenced to the COSMIC database to find hotspot mutations. Variants not defined as hotspot mutations and with a variant allele frequency exceeding 25% in both plasma and matched WBC samples were called as germline variants. Moreover, variants detected in specific genes with matched mutations in WBC were defined as derived from clonal hematopoiesis. Finally, the authors defined cell-free tumor load (cfTL) by tracking the mutant allele frequency (MAF) of the top tumor-derived variant from the first to last timepoint. If MAF decreased to undetectable levels, i.e., 0%, the patient was classified as "molecular response" while continued detection of the top tumor-derived mutation across the longitudinal samples was classified as "molecular progression" (MP) (6).

In addition to the ctDNA analysis, the authors also

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conducted T-cell receptor sequencing of a total of 79 patients (including 23 patients analysed for ctDNA) as well as protein expression analysis (92 proteins, Olink Immuno-Oncology panel) from 28 patients to monitor the peripheral T-cell repertoire as well as proteomic profiling in an attempt to decipher risks of immune-related toxicities (6).

By using the elegant approach of combining ctDNA-sequencing, WBC sequencing and tumor tissue sequencing, the authors identified tumor-derived mutations in 26 patients. These 26 patients were included for analysis of cfTL to classify molecular response (MR). Six out of 26 patients were classified as "MR" with an interval of 2 to 15 weeks. Thirteen out of 26 patients were classified as "MP" while the remaining seven patients displayed a transient response (TR) followed by recurrence. Interestingly, MR did not correlate significantly with radiographic response, neither first radiographic response nor best overall radiographic response. However, patients with MR, or TR, displayed significantly prolonged progression-free survival (PFS) [18.51 (MR), 17.82 (TR), 2.7 (MP) months, respectively] and overall survival (OS) [18.51 (MR), 23.01 (TR), 5.75 (MP) month, respectively] compared to patients with MP. The significant changes in survival were not impacted by whether the patients received ICI monotherapy or a combination with chemotherapy, or by PD-L1 expression (6).

In addition, the authors conducted longitudinal T-cell receptor (TCR)-sequencing in 79 patients from two independent cohorts. The results did not reveal any significant changes in TCR clonotype in relation to MR or to clinical outcome. The authors did, however, observe changes in TCR clonotypes, including multiple CDR3 clusters, in patients developing immune-related adverse events (irAE) during treatment (n=17) (6).

Finally, the authors conducted protein analysis in longitudinal samples from 28 patients using the Olink Immuno-Oncology panel. This analysis revealed significant changes on-treatment of multiple proteins related to the adaptive immune system, including IFN-gamma, TNF, IL10, GZMA, GZMH, CXCL9 and PDCD1 (6).

The study conducted by Murray *et al.* brings multiple valuable findings. Firstly, the combination of ctDNA sequencing with WBC sequencing and tumor tissue sequencing is elegant and provides a solid foundation for the interpretation of the presented results. Despite a limited number of patients, molecular responders (clearance of cfTL) associated with prolonged PFS and OS, and in contrast, molecular progressors associated with shortened PFS and OS. Moreover, it is noteworthy that the results indicate that changes in cfTL does not correlate with radiographic response, emphasizing the need to standardize methods to track circulating tumor DNA in clinical practice for NSCLC patients treated with ICIs or a combination of ICIs and chemotherapy. Finally, the reported changes in TCR clonotypes in patients with irAE, revealed by TCR-sequencing, as well as the observed changes in proteins related to the adaptive immunity warrant additional in-depth investigations (6).

However, as the authors also point out, the study has a few limitations. It is of retrospective nature with a limited number of patients. Despite the small cohort size, the cohort contains multiple therapy regimens. Also, tumor-derived DNA was only detected in 26 out of 30 patients. Moreover, it is difficult to evaluate the potential impact of the reported findings from the TCR-sequencing and protein-analysis although the findings of the TCR-sequencing seems more solid given the larger sample size (n=79 *vs.* n=28) and given that samples originated from two independent cohorts. The protein-analysis was far from systematic, based on a limited panel covering detection of only 92 proteins. It would be highly interesting to use a similar set of longitudinal samples subjected to a comprehensive protein profiling, e.g., through mass spectrometry or using a wider panel, either antibody or aptamer-based, to see whether the proposed proteins would still emerge as top-candidates (6-8).

In summary, the proposed findings warrant validation in larger multicentre cohorts. Nevertheless, the findings based on the multi-sequencing approach of serial liquid biopsies strongly argue for future implementation of serial liquid biopsies as an aid for clinical decision-making. It is, however, imperative that such implementation includes collection of WBCs, and, if possible, tumor tissue, in addition to plasma, to have a reasonable chance to distinguish true tumor-derived mutations from other genetic aberrations, including germline mutations. An optimal tracking of tumor-derived DNA may further argue for access to more systematic sequencing approaches, as reported by Murray *et al.*, rather than the limited panels often used in today's clinical setting (6,9,10). Systematic sequencing approaches are likely to find variants with more abundant MAF at treatment initiation, leading to increased robustness of tumor load tracking over time. Moreover, systematic sequencing approaches in a longitudinal setting may find variants justifying a switch to targeted therapies

following disease progression on ICIs. Finally, a robust tracking of tumor load using liquid biopsies is not only non-invasive for the patient but may also deescalate unnecessary overtreatment of patients responding to ICIs, and hence, minimize the risk of irAE in these patients.

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