Liquid biopsy for tumor mutational burden predicts the effectiveness of atezolizumab in non-small cell lung cancer treatment

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Lung cancer is the principal cause of cancer-related mortality globally. Although there have been significant advances in the treatment of subsets of patients with molecularly defined non-small cell lung cancer (NSCLC), only modest improvements in prognosis have been realized. There is an unfortunate plateau effect associated with traditional chemotherapy. Immunotherapies for advanced NSCLC use immune checkpoint inhibitors (ICIs) to break through this plateau and provide effective treatment. The United States Food and Drug Administration (FDA) approved three ICIs for second-line treatment of NSCLC (nivolumab, pembrolizumab, and atezolizumab), and randomized studies have shown superior overall survival (OS) associated with ICIs, compared to second-line docetaxel (1-4).

Atezolizumab is a humanized and engineered IgG1 monoclonal antibody targeting PD-L1, which can mediate suppression of anticancer immunity by binding to its receptors PD-1 and B7-1 (5). The results of a phase II, randomized study (POPLAR) indicated that atezolizumab improved OS, compared with standard salvage chemotherapy docetaxel, in patients with previously treated NSCLC (6). Atezolizumab monotherapy was also compared with docetaxel in a phase III study (OAK) in which 1,225 patients with PD-L1-unselected advanced NSCLC who were previously treated with one or more platinum-based combination therapies were enrolled (4). In this study, patients were stratified by histology (squamous *vs.* nonsquamous), PD-L1 expression, and prior chemotherapy regimens. Study results indicated that atezolizumab prolonged OS, the primary endpoint, compared with docetaxel regardless of PD-L1 expression [median OS, 13.8 versus 9.6 months; HR, 0.73, 95% confidence interval (CI): 0.62–0.87]. The OAK study was the first trial to show that atezolizumab treatment significantly improved OS, compared with docetaxel, in patients with advanced stage NSCLC. This study also showed a survival benefit associated with atezolizumab, regardless of PD-L1 expression status and histology. Furthermore, patients with tumors expressing increased PD-L1 levels derived the greatest OS benefit from atezolizumab.

Although PD-L1 expression is a useful biomarker for selecting ICI or cytotoxic drugs during NSCLC treatment (3,7), some tumors deemed "PD-L1 positive" do not respond to ICI. Further, some responses do occur in PD-L1 negative tumors treated with ICI. Expression of PD-L1 alone does not completely explain the OS benefit in patients treated with these drugs. Additionally, PD-L1 expression may be limited by tumor heterogeneity and the dynamic nature of the immune microenvironment; consequently, other checkpoint molecules could be explored.

Tumor mutational burden (TMB) is another biomarker associated with clinical efficacy of ICI. Higher nonsynonymous mutation burden in tumors was associated with improved objective response, durable clinical benefit, and progression-free survival (PFS) in patients with NSCLC treated with pembrolizumab (KEYNOTE-001) (8,9). Patients who received nivolumab also exhibited a

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higher response rate and a longer median PFS following first-line NSCLC treatment (CheckMate-026), compared to those who received standard chemotherapy (10). Furthermore, combining nivolumab and ipilimumab resulted in a significantly longer PFS than that observed with chemotherapy alone among patients with NSCLC and a high TMB (Checkmate-227) (11). In these three trials, the level of TMB and the level of PD-L1 expression did not appear to be associated.

Therefore, TMB may have an important impact on understanding patients' responses to ICI therapies, and on the clinical application of these agents. To measure PD-L1 expression and TMB, a sufficiently large volume of tissue must be obtained at biopsy from primary or metastatic lesions. Not all patients will have sufficient tissue or will be able to safety undergo a biopsy. Furthermore, a single point biopsy may only show the genetic heterogeneity of numerous tumor subclones, making evaluation of systemic TMB difficult. Human blood samples contain materials, including cell-free DNA (cfDNA), that originate from different tissues, including cancers. Because the rapid turnover of cancer cells is believed to constantly release tumor-derived nucleic acids and vesicles into the circulation, viable tumor cells can also separate from the tumor and enter the bloodstream. Thus, the ability to detect and characterize circulating tumor DNA (ctDNA) has enabled clinicians to repeatedly and non-invasively examine the dynamic evolution of human cancers (12). Recently, the United States FDA approved the first blood-based assay (cobas *EGFR* Mutation Test v2, Genentech) for detecting mutations in the *EGFR* gene. Because TMB is now measured only using tissue samples, establishing a method for measuring TMB using blood ctDNA should enable us to easily and less-invasively evaluate the effects of ICI on lung cancers.

In the September issue of *Nature Medicine*, Gandara *et al.* reported the clinical benefits of measuring TMB in plasma (bTMB) in patients with NSCLC treated with atezolizumab (13). They retrospectively analyzed two large randomized trials, POPLAR and OAK, as test and validation studies and showed that bTMB reproducibly identified patients with second-line and higher NSCLC who derived clinically significant improvements in PFS from atezolizumab.

The bTMB assay utilizes a hybridization-capture methodology similar to that of the FDA-approved FoundationOne (F1) CDx NGS assay, targeting 1.1 Mb of coding sequence (14). In their study, Gandara *et al.* first performed analytic validation of the bTMB assay to establish both precision of the bTMB score and reliability of the bTMB status, as well as determine the lower limit of tumor content within a cfDNA sample that is needed to construct reproducible bTMB determinations. The bTMB score was determined by identifying all base substitutions present at an allele frequency of ≥0.5% across the coding region of 394 genes and filtering out germline events by comparing against the dbSNP and ExAC databases. The bTMB results obtained from pre-treatment plasma were compared with those from a tissue-based analysis of TMB, obtained from a subset of POPLAR and OAK samples with sufficient tissue for analysis from the same patients. They obtained a positive correlation between tissue TMB (tTMB) and bTMB scores, but it was low (Spearman rank correlation: 0.64; 95% CI: 0.56–0.71). The lower correlation between tTMB and bTMB could be affected by multiple factors, such as fundamental technical differences [the tTMB algorithm considers both single-nucleotide variants (SNVs) and insertions and deletions (indels) at an allele frequency of ≥5%, whereas the bTMB algorithm only considers SNVs but at an allele frequency of ≥0.5%], differences of mutational profile between a single tissue biopsy sample and ctDNA that is released into the blood by metastatic tumors, and differences in the sample characteristics, such as DNA source, collection time, sample type, stage at diagnosis, and tissue purity. Furthermore, to confirm that the variant calls made by the bTMB assay were reliable, individual variant calls made by the bTMB assay were orthogonally validated. For this analysis, they combined clinical samples and cell lines and compared variant calls to FoundationACT (FACT), a previously validated assay that used high-depth sequencing to detect low-abundance variants in cfDNA from plasma. To assess the consistency between these assays, they compared the variant allele frequencies for each matching variant and found that both the assays detected largely the same variants, so this bTMB assay was used in further investigations. Lastly, they assessed the association between clinical outcomes and the bTMB score using 273 baseline plasma samples and the study population from the POPLAR study. Improvement in PFS and OS was observed for all three bTMB cutoff points (≥10, ≥16 and ≥20) relative to the biomarker-evaluable population and the intention-to-treat populations in the POPLAR study. At the bTMB cutoff point of ≥16, the PFS was 0.57 (95% CI: 0.33–0.99).

Following demonstration of bTMB efficiency in the POPLAR study, bTMB analysis was performed using plasma samples from the pivotal OAK trial. Based on the

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technical performance of the bTMB assay at the cutoff point of ≥16 and the stronger PFS treatment effect in the POPLAR study, a cutoff point ≥16 was chosen for confirmatory analysis in the OAK study (16 total mutations of target 1.1 Mb of coding sequence in the bTMB assay corresponded to 14 mutation/Mb). Here 583 patients without *EGFR* or *ALK* alterations had sufficient cfDNA and the bTMB ≥16 subgroup population was 27%. Patients with bTMB ≥16 obtained significant PFS and OS benefit [PFS; HR: 0.65 (95% CI: 0.47–0.92); P=0.013, OS; HR: 0.64 (95% CI: 0.44–0.92); P=0.017] from atezolizumab versus docetaxel. Among patients treated with atezolizumab, the PFS differed between the bTMB ≥16 and bTMB <16 subgroups (HR: 0.65 and HR: 0.98, respectively), whereas the OS benefit did not differ (HR: 0.64 and HR: 0.65, respectively). Although a significant PFS and OS benefit based on expression levels of PD-L1 was observed in patients treated with atezolizumab in the OAK study, significant correlations between increased PD-L1 expression and bTMB was not observed. Patients with bTMB ≥16 and high PD-L1 expression appeared to derive the most clinical benefit from atezolizumab [PFS, HR: 0.38 (95% CI: 0.17–0.85); OS, HR: 0.23 (95% CI: 0.09–0.58)], whereas patients with only high PD-L1 expression or only bTMB ≥16 had PFS HRs of 0.71 (95% CI: 0.43–1.16) or 0.70 (95% CI: 0.48–1.03), respectively. These results indicated that bTMB was a predictive biomarker for PFS in patients with NSCLC who received atezolizumab monotherapy. According to their analysis of these two studies (POPLAR and OAK), bTMB ≥16 is a clinically meaningful cutoff point for NSCLC. Furthermore, bTMB is not correlated with increased PD-L1 expression, as is the case in other trials for TMB (11,12), and independently predicts PFS benefit.

TMB measurement in plasma is precise and reproducible; furthermore, bTMB is related to clinical benefit from ICI therapy. Instead of tissue, using plasma as a DNA source makes the bTMB assay an especially attractive alternative for patients with metastatic NSCLC who are not amenable to biopsy or whose tumor tissue is otherwise unobtainable. Bearing in mind that increased TMB is associated with a greater probability of displaying tumor neoantigens on histocompatibility locus antigen (HLA) molecules on tumor cell surfaces, tumors with increased TMB are more probable to respond to ICI agents because the greater mutation load increases the likelihood of recognition by neoantigen reactive T cells. TMB was previously determined by whole exome sequencing (WES) performed on tumor DNA and matching normal DNA (15-17). Normal germline variations in DNA sequences between individuals should be identified and eliminated from consideration to tabulate only somatic alterations. TMB, which is typically reported as the total number of coding and somatic mutations, can also include insertions and deletions. Theoretically, exonic TMB is best measured by WES because this technique considers the entire exome as the sample. However, because of its higher cost and complexity, TMB by WES is not used routinely in clinical settings for predicting response to ICIs, and its use is mostly restricted to research. Recently, efforts have been made to validate targeted NGS panels against WES data because these panels are regularly used in clinical settings to detect oncogenic mutation (18,19).

Using the F1CDx approach, TMB was defined as the number of base substitutions (including synonymous mutations) present in the coding region of targeted genes. Although germline DNA was not sequenced, filtering for both oncogenic driver events and germline status was performed using public and private variant databases. The total mutations/Mb calculation included both synonymous and nonsynonymous mutations. A bridging formula was required for conversion to number of missense mutations, as determined by WES. F1CDx and MSK-IMPACT, which is another targeted NGS panel, showed that large targeted panels were sufficiently precise for TMB estimation and can predict response to ICI treatment (20,21).

Although bTMB may become a future alternative method of measuring TMB in patients with inadequate tissue, some limitations require attention. *Table 1* shows the trials of ICIs and inter-trial differences in measuring TMB (6,8-11,13,22). Different numbers of samples, different methods for measuring TMB, and different cutoff point values and units were used in each trial. WES was used in CheckMate-026 and KEYNOTE-001, while F1CDx was used in CheckMate-227 and POPLAR. In each trial, several mutations were also excluded from TMB measures. As is the case of bTMB assay in OAK, bTMB calculation was based only on SNVs with exclusion of indels. tTMB and bTMB are new biomarkers with poorly-established standards for determination, so future standardization is warranted. Furthermore, bTMB and tTMB possess different properties because NSCLC features significant intratumoral heterogeneity, and the mutational profile of a single biopsy sample can significantly vary from the net output of ctDNA released into the blood by metastatic tumors. It is unknown which is more useful for predicting treatment efficacy, and further elucidation is needed. All trials that showed TMB

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Table 1 Trials of immune checkpoint inhibitor and differences in measuring tumor mutational burden (TMB)

Variable	CheckMate-026	CheckMate-227	KEYNOTE-001	POPLAR	OAK
Number of samples	312	299	34	92	583
Method	WES	F1CDx	WES	F1CDx	F1CDx (bTMB)
Cut-point of high TMB	243	10	200	16.2	14[a]
Unit	Total somatic missense mt/s	mt/Mb	Total nonsynonymous mt/s	mt/Mb	mt/Mb

[a], bTMB ≥16 equal to ≥14 mt/Mb. WES, whole exome sequencing; F1CDx, FoundationOne CDx; mt, mutations.

efficacy for predicting benefit of ICIs in patients with NSCLC were retrospective analyses or prospective trials later added TMB as a primary endpoint (CheckMate-227). Prospective studies evaluating the usefulness of bTMB are currently ongoing. The Phase III Blood First Assay Screening Trial (B-FAST, NCT03178552) seeks to validate bTMB as a non-invasive biomarker of response to first-line atezolizumab in patients with NSCLC patients, and the phase II Blood First-Line Ready Screening Trial (B-F1RST, NCT02848651) evaluates the effects of atezolizumab monotherapy in patients with first-line NSCLC.

In summary, bTMB measurement is feasible. The cutoff point of ≥16 reproducibly identified patients with an increased benefit of PFS from atezolizumab. We therefore believe that bTMB holds great potential for expanding personalized cancer immunotherapy to even more patients, representing a major advance for the field of liquid biopsy. Furthermore, patients who are suitable for atezolizumab therapy may be selected using a combination of bTMB and PD-L1 expression in the future. TMB itself is a novel type of biomarker with poorly-established standards for determination and reporting. Additional research is required to better understand tTMB and bTMB, and adaptation of the bTMB assay for future molecular diagnostic and therapeutic algorithms for patients with advanced NSCLC may be warranted.

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Footnote

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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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