Editorial Commentary

Blood tumor mutational burden: are we ready for clinical implementation?

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Immunotherapy by using antibodies against the immune checkpoint inhibitor (ICI) PD-1 demonstrated objective durable responses in approximately 20% of patients with advanced non-small-cell lung cancer (NSCLC), melanoma, or renal-cell cancer (1). Later on, the antitumor activity of anti-PD-1 antibody in the treatment of advanced NSCLC was confirmed, showing better efficacy in those patients expressing PD-L1 in at least 50% of tumor cells (2). However, despite this major improvement in the treatment of advanced cancers, immune checkpoint blockade is not successful across all patients or tumor types, with a wide spectrum of response rates, ranging from no response to complete response. Therefore, a great effort to identify robust biomarkers predictive of response to immunotherapy for patient stratification and selection is currently ongoing. The mutational load or tumor mutational burden (TMB) has emerged as a new predictive biomarker for response to checkpoint inhibitors, because it showed a good correlation with response to immunotherapy treatment (3-5). TMB is defined as the total number of somatic mutations per coding area of a tumor genome, although the genes and the size of the sequenced region, as well as the nature of the mutations considered, may vary among the different studies and is currently subjected to debate. Theoretically, tumors with a high TMB are more likely to express neoantigens and to induce a more robust immune response in the presence of ICIs (6). In light of the promising results obtained in several clinical studies, there is an urgent need to move the TMB assays into the clinical practice. Several challenges are in the upfront that may limit the clinical implementation of the TMB, because a reliable and accurate TMB assessment should be guaranteed. Among them, the sample size and the amount and source of available DNA (for example, from formalin-fixed, paraffin-embedded or fresh frozen tissue), the variety of alternative testing platforms, the different bioinformatic pipelines, the definition of cut-offs, the costs, and the need for inter-laboratory standardization.

TMB was initially measured by using next-generation sequencing (NGS) of the whole exome (whole exome sequencing; WES) of the tumor tissue, and it required a matched non-tumor tissue in order to make germline comparisons. This methodology is, however, challenging when intended to implement in a routine clinical setting, due to its high cost, time and bioinformatic complexity. At present, targeted gene panels to determine TMB have been developed, and they constitute an attractive alternative for their use in the routine of molecular pathology laboratories. The implementation of TMB through gene panels reduces the sequencing costs, the DNA input requirements and the turnaround time (TAT). Furthermore, these gene panels produce a deeper coverage compared to WES, potentially improving the sensitivity, which may be very important when the content of tumor cells or the amount of DNA are low. Recent studies have confirmed that TMB measured by NGS cancer gene panels is a predictive biomarker for ICI treatment for NSCLC patients (7-9). Clinically validated

gene panels include Memorial Sloan Kettering Cancer Center's Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) and Foundation One CDx (F1CDx). Commercial gene panels also are available such as TrusSight Oncology 500 from Illumina and Oncomine Tumor Mutation Load Assay from ThermoFisher Scientific. While a variety of such gene panels of differing sizes are offered, there has been intense debate on the ideal sizes or methods of calculating TMB. In a recent study published in *JAMA oncology*, Wang and colleagues (10) used WES data of 9205 NSCLC cancer from The Cancer Genome Atlas (TCGA) to determine the minimum gene panel size and to optimize the cost-benefit ratio. As expected, a larger panel size increases the correlation between the panel- and WES-based TMB along with a decreasing Standard Deviation. This *in silico* analysis showed that a minimum of 150 genes should be covered. Interestingly and as previously reported, the inclusion of synonymous mutation strongly increases the sensitivity of the test when using small (<150 genes) panel size. Panel composition should also be carefully selected to obtain sufficient separation of hypermutated tumors from non-hypermutated tumors. The authors designed a cancer gene panel covered whole exon regions of 150 selected cancer-related genes and called this panel NCC-GP150. The panel was virtually validated using TCGA database. To further test the practicability of NCC-GP150, the authors used a public dataset including 34 patients with NSCLC treated with PD-1 inhibitor pembrolizumab. The progression-free survival was significantly longer in patients with high TMB than in patients with low TMB.

The genetic profiling of tumors involves necessarily the use of tissue biopsies. Nevertheless, the availability of adequate tissue can be a limiting factor, especially for NSCLC patients (11). This is highlighted by the reduced number of patients for whom TMB evaluation on tissue samples was possible in clinical trials [59% in CheckMate 026 (7) and 58% in CheckMate 227 (8)]. In some instances, liquid biopsies are used alternatively to tissue biopsies, and they may be even preferred due to their noninvasive nature. However, the amount of circulating free DNA (cfDNA) varies greatly depending on diverse pathological conditions, such as the type of tumor, progression status, proliferative rate, and therapy regimen of the patient (12). In addition, the fraction of circulating tumor DNA (ctDNA) in total cfDNA is usually low. Therefore, a highly sensitive method would be needed to analyze TMB in cfDNA samples. The ctDNA isolated from blood can be analyzed by different technologies, including allele-specific PCR, digital droplet PCR and panel based-NGS. For TMB, given that the sensitivity of NGS-based technologies is inversely proportional to the number of loci analyzed, the use of gene panels that allow deeper coverage than WES would be more appropriate. Wang *et al.* (10) found that blood TMB (bTMB) can be reliably evaluated by their NGS panel (NCC-GP150) which was showed to have a satisfactory performance as compared to WES. Indeed, bTMB estimates via their panel correlated well with tissue TMB estimates via WES (Spearman correlation, 0.62). Moreover, high bTMB was associated with superior progression-free survival and objective response rates to ICIs. The authors validated their panel not only analytically, but also clinically. TMB evaluation on ctDNA is thus a very attractive tool, as it is non (less) invasive. However, very similar challenges, as for tissue, are associated with bTMB implementation in a routine setting; testing platforms and panels, bioinformatic pipelines, cut-off definition, costs and the standardization of the method of TMB measurement. In addition, the presumed low frequency of the variants in the ctDNA can result in a higher rate of false negatives and, therefore, requires significantly greater technical effort and expertise to obtain reliable results. Furthermore, it is known that early stage tumors release very little ctDNA, so some technical challenges still need to be resolved. The authors recognized different limiting factors: (I) the clinical validation was retrospective on a small cohort (n=50); (II) the clinical cohort was obtained from different and heterogeneous trials; (III) the use of TCGA data for virtual validation but a Chinese cohort for technical and clinical validation. However, the study of Wang *et al.* (10) is an additional contribution for TMB as a predictive biomarker for ICI treatment.

In conclusion, TMB and bTMB are promising tools as potential biomarkers in NSCLC. However, the harmonization of the TMB measurement across platforms, as well as the definition of the thresholds for each type of tumor are necessary steps to implement into the clinic the measurement of TMB as part of a personalized medicine approach (13). Moreover, a standardization of the TMB measurement method would determine a greater sensitivity, reliability, reproducibility and robustness, which is essential to guarantee its clinical usefulness.

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Footnote

Conflicts of Interest: 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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