Review Article

Current views on tumor mutational burden in patients with non-small cell lung cancer treated by immune checkpoint inhibitors

Léa Berland[1,2], Simon Heeke[3], Olivier Humbert[4], Adam Macocco[1,2], Elodie Long-Mira[1,2], Sandra Lassalle[1,2], Virginie Lespinet-Fabre[1], Salomé Lalvée[1], Olivier Bordone[1], Charlotte Cohen[5], Sylvie Leroy[6], Véronique Hofman[1,2,5], Paul Hofman[1,2,7], Marius Ilié[1,2,7]

[1]Laboratory of Clinical and Experimental Pathology, Pasteur Hospital, University Hospital Federation OncoAge, [2]UFR Médicine, [3]Institute of Research on Cancer and Ageing of Nice (IRCAN), University Hospital Federation OncoAge, CNRS, INSERM, [4]Department of Nuclear Medicine, Antoine Lacassagne Comprehensive Cancer Center, Université Côte d'Azur, Nice, France; [5]Department of Thoracic Surgery, [6]Department of Pulmonary Medicine and Oncology, [7]Hospital-Related Biobank (BB-0033-00025), Pasteur Hospital, University Hospital Federation OncoAge, Université Côte d'Azur, Nice, France

Contributions: (I) Conception and design: M Ilié; (II) Administrative support: M Ilié, P Hofman; (III) Provision of study materials or patients: M Ilié, S Heeke, L Berland, O Humbert, A Macocco, E Long-Mira, S Lassalle, C Cohen, S Leroy, V Hofman, P Hofman; (IV) Collection and assembly of data: L Berland, M Ilié; (V) Data analysis and interpretation: L Berland, S Heeke, M Ilié; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Marius Ilié. Laboratory of Clinical and Experimental Pathology, Pasteur Hospital, 30 Voie Romaine, BP69, 06001 Nice cedex 01, France. Email: ilie.m@chu-nice.fr.

Abstract: In the last few years, the treatment of patients with non-small cell lung cancer (NSCLC) has impressively benefitted from immunotherapy, in particular from the inhibition of immune checkpoints such as programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1). However, despite the significant survival benefit for some patients with advanced NSCLC, the objective response rates (ORRs) remain relatively low no more than 20–30% with a large proportion of patients demonstrating primary resistance. Although the selection of NSCLC patients for the first-line treatment is currently guided by the expression of PD-L1 in tumor cells as detected by immunohistochemistry, this is not the case for the second-line setting. Moreover, the sensitivity and specificity of PD-L1 expression is modest which has prompted the search for additional predictive biomarkers. In this context, the assessment of the tumor mutational burden (TMB), defined as the total number of nonsynonymous mutations in the coding regions of genes, has recently emerged as an additional powerful biomarker to select patients for immunotherapy. The purpose of our review is to highlight the recent advances as well as the challenges and perspectives in the field of TMB and immunotherapy for patients with NSCLC.

Keywords: Tumor mutational burden (TMB); immunotherapy; non-small cell lung cancer (NSCLC); tumor tissue; circulating tumor DNA

Submitted Oct 04, 2018. Accepted for publication Nov 19, 2018.
doi: 10.21037/jtd.2018.11.102
View this article at: http://dx.doi.org/10.21037/jtd.2018.11.102

Definition of tumor mutational burden (TMB)

TMB can be defined as the total number of nonsynonymous mutations in the tumor exome (1). Tumor cells are genetically unstable and harbor high levels of somatic mutations which may result in the expression of neoantigens, that are not subject to immune tolerance (2). The presentation of tumor-specific neoantigens on major histocompatibility complex molecules is essential for the recognition of tumors by the T-cells. Patient-specific neoantigens that develop following somatic mutations have been shown to induce a T-cell response (3,4). The

production of neoantigens is not necessarily correlated to a higher proportion of somatic mutations, but the latter may increase the odds of developing neoantigens (5). The prevalence of somatic mutations is considerably variable between different types of tumor, with non-small cell lung cancer (NSCLC) having among the highest mutation frequency of 0.1 to 100 mut/Mb (1,6).

As such, tumors with high nonsynonymous TMB express large numbers of abnormal proteins, which are recognized by the immune system as neoantigens (7). These tumors may be eligible to successful immune-mediated destruction if their T-cell-dependent immune responses against tumor cells could be overreached (8).

With the growing interest in TMB, different studies investigated the possible links between TMB and characteristics of NSCLC patients. As such, TMB correlates with patients' gender, being higher in men, and tobacco consumption, with higher levels identified in active smokers (9). Indeed, dominant C > A mutations, caused by tobacco smoking and exposure to polycyclic aromatic hydrocarbons are responsible for DNA damage, and increase the risk of lung cancer by increasing TMB and therefore the possibility of having a mutant driver compared to non-smoking controls for which the presence of a mutant oncogene driver is sufficient to induce tumorigenesis. Microsatellite instability or functional loss of DNA repair genes lead to high TMB, but the biologic significance of such event still remains unclear (5).

Other studies have also shown that TMB may be associated with certain mutations regularly found in NSCLCs. In a large dataset, the mean TMB and the proportion of patients with a TMB of >10 or >20 mut/Mb was significantly higher for *KRAS* mutated patients (10.3 mut/Mb) than in *EGFR*, *ALK*, *ROS1* or *MET* exon 14 mutated patients (3.1 to 6.2 mut/Mb). This low TMB could be related to the low efficacy of immune checkpoint inhibitors (ICIs) in these NSCLC cases (10).

Mean TMB was similar for *BRAF* mutated patients compared to *KRAS* mutated patients (9.7 versus 10.3 mut/Mb), and all adenocarcinoma patients show a comparable TMB to these patient groups (mean 9.1 mut/Mb), whereas patients with squamous cell carcinoma have a relatively higher mean TMB (11.3 mut/Mb) (11).

Clinical utility of TMB in patients with NSCLC treated by immunotherapy

In the last decade, immunotherapy using ICIs such as monoclonal antibodies targeting programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1) has become a standard of care treatment for patients with advanced or metastatic NSCLC in first and later treatment lines (12). However, the overall response rate (ORR) with ICIs barely reaches 20% and a considerable proportion of patients will undergo disease progression within the first weeks of treatment (13). Moreover, the optimal selection of NSCLC patients who will benefit most from treatment with ICIs is far from being well-defined (14). The PD-L1 expression as a predictive biomarker in NSCLC patients has shown some value for predicting response to ICIs in some clinical trials. While the efficacy on overall survival (OS) of nivolumab and atezolizumab was independent from PD-L1 expression, pembrolizumab was associated with prolonged OS in comparison with chemotherapy in the first-line treatment of advanced NSCLC with a PD-L1 expression ≥50% of tumor cells and in second-line treatment of tumors with a PD-L1 expression ≥1% of tumor cells. In addition, durvalumab was responsible for a longer progression-free survival (PFS) in comparison with placebo after chemoradiotherapy in patients with stage III NSCLC independently of the PD-L1 expression (15). Finally, neoadjuvant administration of nivolumab in patients with early-stage NSCLC was associated with few immediate adverse events, did not delay planned surgery, and led to a major pathological response regardless of PD-L1 expression (16). Therefore, the use of PD-L1 expression as a robust predictive biomarker has been confounded with a number of biological and technological variables which has prompted the establishment of improved biomarkers for better stratification of NSCLC patients treated by ICIs (17,18). Alongside PD-L1 expression, FDA only approved mismatch repair deficiency as a predictive biomarker for ICIs blockade with pembrolizumab (19).

Most recently, several NSCLC clinical trials have provided evidence that TMB correlates with the clinical response of ICIs, offering a new perspective for predicting ICIs treatment outcomes of NSCLC patients in the near future.

The first evidence of correlation between high nonsynonymous mutational burden and improved objective response rate (ORR), durable clinical benefit (DCB), and PFS obtained with immunotherapy was demonstrated by using whole-exome sequencing (WES) in advanced NSCLC from two independent retrospective cohorts of patients treated with pembrolizumab, and their matched normal DNA (8). Patients with a partial response or stable disease for more than six months showed a median number of non-synonymous mutations

of 302 versus 148 in patients with no DCB. TMB was higher in advanced NSCLC patients with a DCB than in those with an NDCB (median, 8.5 *vs.* 6.6 mut/Mb). TMB was also greater in patients with a complete response (8.5 mut/Mb) or partial response versus those with stable disease and those with progressive disease (6.6 mut/Mb for both stable disease and progressive disease) (8,20).

In the open phase III trial CheckMate-026 which compared nivolumab to platinum-based chemotherapy, less than 100 mut/Mb was defined as low TMB, a medium TMB was between 100 and 242 mut/Mb and a high TMB was considered beyond 243 mut/Mb. In the third category the median PFS was longer (9.7 *vs.* 5.8 months) and the ORR was higher in the nivolumab group than in chemotherapy group (47% *vs.* 28%). In addition, patients in the nivolumab group with both high TMB and a PD-L1 expression ≥50% TCs had a higher response rate (75%) than those with only one of these factors (32% among patients with a high TMB only and 34% among those with a PD-L1 expression level of ≥50% only) or neither factor (16%) (21).

Furthermore, the CheckMate-227 study evaluated multiple hypotheses in a population of patients with advanced NSCLC based on the efficacy of nivolumab or the combination of nivolumab and ipilimumab as first-line therapy. By grouping patients with ≥13 mut/Mb and a PD-L1 expression ≥1% TCs, this study found no significant difference in PFS between patients treated with chemotherapy (5.6 months) and those treated with nivolumab alone (4.2 months) (HR, 0.95; 97.5% CI, 0.61 to 1.48; P=0.78) (22). However, this study provided evidence that TMB correlates with the clinical response to the combination of nivolumab and ipilimumab and demonstrated a significant primary endpoint PFS benefit in the TMB group, whereas PFS benefit was seen regardless of the PD-L1 status of all patients suggesting that TMB may be more robust than the PD-L1 expression to predict clinical response following treatment with combination regimens (22). These findings were independently validated in a retrospective study of 75 patients with NSCLC treated with nivolumab and ipilimumab as part of the CheckMate-012 study, suggesting that the combination immunotherapy may be particularly effective in patients with high TMB but may be insufficient to overcome the negative predictive impact of low TMB (23).

Finally, a large retrospective analysis across seven atezolizumab monotherapy trials showed that high TMB (≥16 mut/Mb) assessed with the FoundationOne CDx (F1CDx; Foundation Medicine, Cambridge, USA) assay in tumor tissue is associated with improved ORR and duration of response across different advanced solid tumors, including NSCLC, and several lines of therapy. Importantly, the significant association between TMB and atezolizumab efficacy occurred regardless of the PD-L1 status in NSCLC patients (24,25).

Overall, TMB appears as a novel effective biomarker, irrespective of the PD-L1 expression, for predicting response to ICIs as monotherapy, but it can also play a key role in the choice of a combination of ICIs in patients with advanced NSCLC.

In the future, immunotherapy may also include perioperative use as highlighted by two recent studies which explored the clinical value of TMB in resectable NSCLC patients. Induction of a systemic immune response before resection of the primary tumor could lead to immunologic memory that may improve protection against tumor growth and recurrence over a long term (26). In a pilot study of 21 patients with early-stage NSCLC treated with nivolumab before surgery, 45% of the patients achieved a major histological response at the time of the planned surgical resection, which was highly associated with an increased TMB (sequence alterations; mean, 311±55 *vs.* 74±60, P=0.01) (27).

A second study evaluated the clinical value of TMB from the Lung Adjuvant Cisplatin Evaluation (LACE)-Bio-II study, which analyzed 908 specimens of early-stage NSCLC from patients treated in three adjuvant clinical trials (28). The TMB was measured using a targeted panel of 1,538 genes by analysis of the TCGA atlas and was significantly correlated with the TMB calculated through WES. In this study, tumors with a high nonsynonymous TMB >8 mut/Mb had a favorable outcome in terms of DFS, OS and lung cancer-specific survival. However, the predictive effect of TMB on response to chemotherapy was only modest in terms of survival (28). In another cohort, NSCLC patients with lower TMB had improved responses, but the correlations between TMB and response to chemotherapy or survival needs further independent validation (21). Moreover, these findings raise several important questions on whether cancer recurrence will be prevented, whether recurrent tumr clones will still be responsive to ICIs, and whether molecular resistance pathways will emerge within recurrent tumors (26).

Measurement of TMB in tumor tissue

WES is a comprehensive method, considered as the

gold standard method for assessing TMB. Using NGS technology, WES allows the detection of somatic mutations presents within the entire exome (~2% of the human genome, i.e., 30 to 50 Mb of coding sequences) (29). WES provides a comprehensive picture of all mutations that may have a role in tumor progression at a lower cost than whole genome sequencing. However, despite the proven utility of WES in measuring TMB and predicting response to PD-1/PD-L1 blockade, it has several limitations such as the high cost, large input DNA amount, or workflow and analysis complexity unfitted for the clinical setting. Thus, protocols for optimal WES analysis require a minimum amount of tumor material, between 150 and 250 ng of genomic DNA, which may be difficult to obtain on biopsies that are too small or do not contain enough tumor cells (30). Also, it is recommended to sequence in parallel non-tumor tissue in order to be able to identify tumor-specific variants, which necessarily leads to increased costs and additional required material not available in every clinical scenario (31).

Moreover, the ability of WES technology to detect DNA alterations depends of the sequencing depth and the variant frequency within the tumor. At a coverage of 50×, 95% of short insertions and deletion and single nucleotides variants can be detected with a variant allele frequency of only 15% (32). However, these numbers do not take into consideration the intra-tumor heterogeneity and aneuploidy as well as the contamination with non-tumor cells. Thus, samples with low purity and high tumor heterogeneity will require deeper sequencing to maintain high sensitivity (33).

Thus, as for the whole genome sequencing, the WES analysis can be difficult to implement in routine practice due to its high cost or turnaround time and technological complexity. The targeted gene panel sequencing was recently evaluated as an alternative approach for clinical use (30). Targeted mutation analysis of selected "driver" genes made the promise to deliver results at a lower cost and decreased turnaround time. In addition, targeted gene panels can generate deeper sequencing compared to WES and could, while using less amounts of DNA, improve mutation detection sensitivity (23).

A recent study evaluated TMB results from over 2,000 Japanese patients with solid tumors using WES and a targeted gene panel (409 genes). TMB levels were divided into three groups: low (≤5 mutations/Mb), high (≥20 mutations/Mb), and intermediate (the margin between these intervals) (34). The TMBs calculated using the two platforms was highly correlated. However, the estimated TMB based on panel sequencing was significantly overestimated in samples harboring lower TMB levels, suggesting that the available targeted gene panel could accurately estimate TMB in tumors with high-frequency mutations. Overall, the TMB analysis using the targeted gene panel was strongly correlated with the WES platform (34).

Another retrospective study compared the performance of both WES and a custom hybridization capture-based NGS assay (MSK-IMPACT, 341 genes; Memorial Sloan Kettering Cancer Center, New York, USA) to measure TMB in 49 patients with advanced NSCLC treated with PD-1/PD-L1 inhibitors (20). TMB assessed by targeted NGS was highly correlated with TMB assessed by WES, and was higher in patients with DCB or complete/partial response than in patients with no durable benefit or stable/progressive disease. These results demonstrated that targeted NGS may accurately estimate TMB, while high TMB may improve benefit to ICIs. Interestingly, TMB did not correlate with PD-L1 expression, and both biomarkers had similar predictive capacity, suggesting that the integration of both TMB and PD-L1 expression into multivariable predictive clinical models should result in increased predictive power to estimate the response to immunotherapy (20).

As described above, PFS was significantly longer with first-line nivolumab plus ipilimumab than with chemotherapy among patients with NSCLC and a high TMB measured with the F1CDx assay in tumor specimens (22). F1CDx is a NGS-based *in vitro* diagnostic device designed to detect substitutions, insertion and deletion alterations (indels), and copy number alterations in 324 genes and selected gene rearrangements, as well as genomic signatures including microsatellite instability and TMB using DNA isolated from formalin-fixed paraffin embedded (FFPE) tumor tissue specimens (35).

Furthermore, the performance of a commercially available targeted NGS panel and workflow for TMB analysis (Oncomine™ Mutation Load Research Assay, TML; Thermo Fisher Scientific, Waltham, USA) interrogating 409 cancer-related genes, spanning ~1.7 Mb of the genome, was recently reported in a set of 30 FFPE tumor samples including colon, renal, gastric, endometrial, and lung tissues (36). TMB was measured by counting somatic single-base substitutions per Mb at ≥10% allele frequency in single, non-matched, tumor DNA samples. This study was the first to demonstrate the feasibility of a commercial targeted NGS panel and data analysis pipeline for TMB evaluation in clinical FFPE tumor samples (36).

Overall, TMB estimation through targeted gene

sequencing shows up as an interesting effective option in contrast to WES, with reduced costs and turnaround time required for investigation, which are essential criteria to consider for the transfer in the clinical setting of these technologies.

Assessment of TMB in circulating tumor DNA

While TMB analysis is generally performed on tumor tissue DNA, it is noteworthy that the TMB can also be evaluated from circulating tumor DNA (ctDNA) (37). Initial studies have investigated the association between the hypermutated blood-derived ctDNA and the response to ICIs, demonstrating a significant PFS benefit in patients with high levels of genomic alterations (38).

Furthermore, Fabrizio *et al.* recently described an assay to calculate TMB from ctDNA in the blood (bTMB; i.e., 394 genes), compared TMB between tissue (tTMB) and blood (bTMB), and evaluated variant-level concordance between the bTMB assay and a CLIA-validated ctDNA assay, FoundationACT (FACT; i.e., 62 genes) (39). They further demonstrated the clinical utility of the bTMB assay across a retrospective study of 794 NSCLC patients from two randomized clinical trials evaluating PFS and OS between atezolizumab and chemotherapy (39). This pilot study of 259 patients enrolled in the OAK and POPLAR trials demonstrated an overall agreement of 81.5 % and a positive percent agreement of 61.3% between the 394-gene blood panel and the FoundationOne panel in tumor tissue. However, when bTMB was restricted to the 62 gene FACT assay, the positive percent agreement decreased to 17%, suggesting that a sufficiently sized panel is required to sensitively identify patients with high TMB. In addition, patients with bTMB ≥14 mut/Mb were considered high and demonstrated significant enrichment for both PFS and OS in the phase 2 POPLAR study evaluating atezolizumab versus chemotherapy. Within POPLAR, the PFS HR for bTMB high patient was 0.57. Applying the bTMB cutoff of 14 mut/Mb, the panel was validated in the OAK cohort population, while the PFS HR was 0.65. Moreover, when samples demonstrated high bTMB, the overlap of TMB variants between blood and tissue was 70%. Blood samples tested with both bTMB and FACT assays revealed that 93% of variants were detected in both assays across overlapping regions, with ≥99% of variants detected in both platforms with variant allele frequency ≥1% (39). In addition, the clinical validation of bTMB was recently reported in a prospective, randomized phase III clinical trial, BFAST, evaluating atezolizumab versus chemotherapy as first line in advanced NSCLC patients (40). This study included 583 samples from the OAK trial and 211 samples from the POPLAR study. In both study populations, a bTMB ≥16 mut/Mb was associated to increased PFS and OS when patient received atezolizumab compared to docetaxel, regardless of the PD-L1 status in tumor tissue.

Interestingly, this study also showed that tTMB and bTMB, while being positively correlated, had a positive percentage of agreement of only 64%. The assays detected overlapping but not identical variants in the DNA isolated from asynchronous matching plasma and tumor tissue samples, suggesting that tumor heterogeneity may be the main source of discordances.

The development of ultrasensitive technologies may pave the way for routine detection of low allelic frequency mutations, e.g., for detection of minimal TMB in ctDNA (41). For instance, Newman *et al.* used the CAncer Personalized Profiling by deep Sequencing (CAPP-Seq) method, an ultrasensitive technology optimized for low DNA inputs able to enrich 139 genes frequently mutated in solid tumors, with a coverage of approximately 125 kb by NGS (41). This study evaluated the relationship between the number of detected mutations by WES and CAPP-Seq. To infer the number of non-synonymous mutations that the CAPP-Seq method was expected to find in tumor tissue, the number of mutations identified in plasma DNA was considered the reference. Thus, there were a strong positive correlation between the WES data and those obtained by CAPP-Seq (r=0.93). A TMB of ~200 mutations, considered as the standard for high TMB measured by WES, corresponded to ≥5 mutations detected with CAPP-Seq. Thus, CAPP-Seq may achieve robust assessment of bTMB and can be tuned to deliver suitable sensitivity and specificity (41).

Overall, the assessment of TMB in ctDNA represents an appealing non-invasive option, allowing patients who cannot benefit from tissue biopsy, in light of restricted tumor material or excessively degraded health state, a long-lasting follow-up when treated by ICIs. However, this approach comes with several disadvantages; the main one remains that ctDNA is found in very small quantities, and indistinguishable from circulating non-tumor DNA. Another parameter that could potentially have consequences on the analysis of the bTMB would be the false positive plasma genotyping, due to the presence of non-tumor mutations harbored by hematopoietic cells (42,43). It seems that most *JAK2* mutations, some *TP53* mutations and

rare *KRAS* mutations detected in cfDNA are not derived from the tumor but from clonal hematopoiesis (42). We can reasonably imagine that these observations could have an effect in the TMB calculation, even more when using targeted gene panels.

Additional work is needed to better understand the dynamics and biology of bTMB and its relevance within the context of immunotherapy for NSCLC patients.

Current challenges for TMB analysis

Tumor tissue biopsies used for TMB analysis are often fixed with formaldehyde to preserve tissue and cell morphology. However, this fixative is known to induce various crosslinks which are a main source of sequencing artifacts, notably through DNA fragmentation, denaturation and cytosine deamination. It may be interesting to carry out a specific preparation of samples to minimize these artefacts which may complicate the analysis of NGS results, in particular for low frequency mutations. For instance, microdissection for tumor enrichment or decross-linking by heat treatment prior to DNA extraction may be used to limit such artefacts (44).

NGS technology has enabled the rapid development of TMB analysis. However, certain limitations persist, in particular due to the time needed to carry out these analyses and the quantities of material required. Thus, in 2018, the time needed to perform a TMB analysis was between 1 to 2 weeks, with a cost of 2,500 to 5,000 US dollars, although we can hope that this price will decrease with time. NGS technology is thus 5 to 10 times more expensive than PD-L1 immunohistochemical analysis and uses 10 times more equipment. For example, the FoundationOne assay, one FFPE block or 10 unstained tumor slides cut at 4–5 µm thickness with a minimum of 25 mm^2 surface area and at least 20% of tumor, are required (35).

Furthermore, it is not always possible to perform a TMB analysis for a patient; as seen above, this requires sufficient quality and quantity of material. The percentage of patients enrolled in clinical trials is lower for TMB than for PD-L1. For example, in the Checkmate 568 trial, 88% of patients were evaluable for PD-L1 but only 34% were evaluable for TMB (20,22,40).

Even when the sample's content used for TMB analysis is low in tumor cells (≤10%), it is presently conceivable to achieve greater sensitivity by using targeted platforms and all the more particularly when the coverage is expanded (×500 or ×1,000). Greater coverage is achieved in targeted panels rather than with WES. For example, Rivzi *et al.* achieved an average sequencing coverage of 232× using WES and 744× using the MSK-IMPACT panel (8,20).

To determine the TMB in NSCLC patients, several panel sizes were investigated. The most commonly used, the MSK-IMPACT panel, includes 468 genes. Three other panels have also recently been marketed; the FoundationOne panel, targeting 315 genes; the Oncomine Tumor Mutation Load Assay (409 genes) and the TruSight Tumor 170 (170 genes). However, the uncertainty area associated with the TMB estimation increases rapidly when the panel size decreases; similarly, the coefficient of variation increases rapidly when the targeted gene panel is less than 1–1.5 Mb, which is the minimum size required for a panel of more than 300 genes (30,45).

Lung tumor is one of the two cancer types with the highest TMB with a median TMB of 7.2 mut/Mb across more than 18,000 lung cancer cases, with approximately 12% of the patients showing more than 20 mut/Mb. The WES study from Rizvi *et al.* defined the cut-off point that combined maximal sensitivity (100%) with maximal specificity (67%) at 178 non-synonymous mutations in advanced NSCLC patients (8). For example, PFS among advanced NSCLC patients with a high TMB greater than or equal to 10 mut/Mb was significantly longer with nivolumab plus ipilimumab than with chemotherapy (22). Overall, three TMB categories emerge for tumor classification from various studies. The first corresponds to tumors with a significant TMB (>10 mut/Mb), the second includes tumors with low TMB (<5 mut/Mb) and the last corresponds to tumors that do not fit into either of these two categories. This third "class" of tumors reflects the need to validate an optimal cutoff to reduce this zone of uncertainty. Furthermore, TMB analysis is not standardized for different tests and the cutoff for positivity varies between ≥7.4 to ≥20 mut/Mb when measured by NGS. More prospective clinical trials are needed to correctly determine the threshold. Moreover, the harmonization between thresholds with the different assays remains a key question.

In addition, TMB does not represent direct proof of immunogenicity and does not precisely foresee the dynamic immune reaction (5). Because TMB is a metric variable, a cutoff score must be set as a predictive objective criterion. Until the biologic links between TMB and immunogenicity are understood, any cutoff score remains arbitrary and should be interpreted with caution in the clinic (5).

Furthermore, the production of neoantigens is not linked to germline and non-synonymous mutations, which are

therefore excluded from the TMB calculation, but rather to the presence of non-synonymous mutations. To eliminate the biases due to these non-synonymous and germline mutations, the ideal situation would be to compare these results with the TMB measured on non-tumor samples. However, this would require an additional amount of tissue, and it seems difficult to set up in clinical practice. Various solutions have been proposed to minimize these biases; compare the patient data obtained with databases containing the most common germline and synonyms mutations. However, to be effective in all patients, these databases must be sufficiently representative of the different ethnic groups. Patients with unusual ethnic backgrounds will therefore be at risk for false positive results. Garofalo *et al.* suggest that genomic data should be analyzed by clinicians to reduce the number of false positives, but this manual filtering is time consuming in a routine clinical setting (46).

The inclusion of synonymous and germline mutations in the TMB analysis, or the exclusion of variants known to be oncogenic drivers and germline polymorphisms may explain the variability between studies. Thus, there is a need of standardization to compare the results obtained in different studies. In the study conducted by Qiu *et al.* the need for standardization was demonstrated by the presence of a significant gap between the numbers of mutations reported by different suppliers. The aligner used to align reads to a reference genome and, more importantly, the variant caller or parameters that are used to identify single-nucleotide variants are responsible of modification in the mutation rates in bioinformatics pipelines. The raw data (FASTQ files) obtained from the different WES commercial sources with the same data analysis pipeline [Genome Analysis Toolkit (GATK)/Mutect-based pipeline] gave more consistent results (47).

Finally, guidelines regarding turnaround times for predictive assays recommend 3 workdays from a request for testing to receipt by a reference laboratory and testing results within 10 workdays (48). There are questions about the possibility of clinical routine transfer of the different TMB assays within the recommended turnaround times.

Perspectives

TMB is not the only parameter bound to the responsiveness of immunotherapy and other biological criteria ought to likewise be considered.

TMB includes both clonal (present throughout the tumor) and subclonal (present in only part of the tumor cells) neoantigens (49). It has been demonstrated that especially tumors with a high TMB as well as low neoantigens subclonal fraction (<5%) had the most DCB with pembrolizumab (49). In a series of 31 tumors from NSCLC patients treated with pembrolizumab, only clonal and not subclonal neoantigens were recognized by T-cells. This aspect is not negligible since chemotherapy seems to increase TMB as well as the proportion of subclonal neoantigens; which, as mentioned above, are not recognized by the T-cells (49). A recent study identified genomic correlates of response to ICIs beyond mutational burden, including somatic events in individual driver genes, certain global mutational signatures, and specific HLA-restricted neoantigens, while the response rate was higher when dealing with tumors with clonal mutations (i.e., less intra-tumoral heterogeneity) (50).

Moreover, it is necessary to consider not only the number of new neoantigens, but also their "fitness". This will be possible by using information on the frequency of a clone, but also T-cell recognition probability and by using the amplitude of MHC presentation (51).

Furthermore, the impact of TMB on outcomes of patients treated with targeted therapies has been recently explored. TMB seems to be negatively associated with clinical outcomes in metastatic *EGFR* mutant NSCLC patients treated with *EGFR* inhibitors, suggesting the need for combining predictive testing for *EGFR* mutant status along with TMB analysis in these patients (52).

Finally, although immunotherapy development for SCLC has got behind that for NSCLC, and the efficacy appears to be lower than for NSCLC, recent encouraging results have been demonstrated with combinatorial ICIs in patients with small-cell lung cancer harboring high TMB (53,54).

Conclusions

Immunotherapy treatments have revolutionized the management of patients with NSCLC. However, the main difficulty remains in the decision to assign these treatments to a patient, and to determine early their response. The relative inability of PD-L1 immunohistochemical analysis in these areas has demonstrated the need to develop new predictive biomarkers; hence the growing interest in TMB analysis. Thus, based on the current state-of-the-art TMB seems to be an interesting predictive biomarker and it appears as an essential parameter in future approaches for selecting patients who may benefit from immunotherapy.

Nonetheless, TMB currently is used as a complementary predictive tool without sufficient power to replace PD-L1 testing in the routine clinical setting, at least not before solving the diagnostic complexity, the limitation of sample resources, costs and potential delays in patient care.

Acknowledgements

Funding: This work was supported by the French Government (National Research Agency, ANR) through the "Investments for the Future" LABEX SIGNALIFE: program reference # ANR-11-LABX-0028-01, the Conseil Departemental des Alpes Maritimes, the Ligue Departementale contre le Cancer, the Canceropole PACA and the Association for Cancer Research (ARC).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. Nature 2013;500:415-21.
2. Gubin MM, Artyomov MN, Mardis ER, et al. Tumor neoantigens: building a framework for personalized cancer immunotherapy. J Clin Invest 2015;125:3413-21.
3. Castle JC, Kreiter S, Diekmann J, et al. Exploiting the mutanome for tumor vaccination. Cancer Res 2012;72:1081-91.
4. van Rooij N, van Buuren MM, Philips D, et al. Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol 2013;31:e439-42.
5. Goto Y. Tumor Mutation Burden: Is It Ready for the Clinic? J Clin Oncol 2018:JCO2018793398.
6. Lawrence MS, Stojanov P, Polak P, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 2013;499:214-8.
7. Braun DA, Burke KP, Van Allen EM. Genomic Approaches to Understanding Response and Resistance to Immunotherapy. Clin Cancer Res 2016;22:5642-50.
8. Rizvi NA, Hellmann MD, Snyder A, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 2015;348:124-8.
9. Alexandrov LB, Ju YS, Haase K, et al. Mutational signatures associated with tobacco smoking in human cancer. Science 2016;354:618-22.
10. Schrock AB, Li SD, Frampton GM, et al. Pulmonary Sarcomatoid Carcinomas Commonly Harbor Either Potentially Targetable Genomic Alterations or High Tumor Mutational Burden as Observed by Comprehensive Genomic Profiling. J Thorac Oncol 2017;12:932-42.
11. Skoulidis F, Goldberg ME, Greenawalt DM, et al. STK11/LKB1 Mutations and PD-1 Inhibitor Resistance in KRAS-Mutant Lung Adenocarcinoma. Cancer Discov 2018;8:822-35.
12. Dempke WCM, Fenchel K, Dale SP. Programmed cell death ligand-1 (PD-L1) as a biomarker for nonsmall cell lung cancer (NSCLC) treatment—are we barking up the wrong tree? Transl Lung Cancer Res 2018;7:S275-9.
13. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. Science 2018;359:1350-5.
14. Heeke S, Hofman P. Tumor mutational burden assessment as a predictive biomarker for immunotherapy in lung cancer patients: getting ready for prime-time or not? Transl Lung Cancer Res 2018;7:631-8.
15. Antonia SJ, Villegas A, Daniel D, et al. Durvalumab after Chemoradiotherapy in Stage III Non-Small-Cell Lung Cancer. N Engl J Med 2017;377:1919-29.
16. Forde PM, Chaft JE, Smith KN, et al. Neoadjuvant PD-1 Blockade in Resectable Lung Cancer. N Engl J Med 2018;378:1976-86.
17. Shukuya T, Carbone DP. Predictive Markers for the Efficacy of Anti-PD-1/PD-L1 Antibodies in Lung Cancer. J Thorac Oncol 2016;11:976-88.
18. Ilie M, Hofman V, Dietel M, et al. Assessment of the PD-L1 status by immunohistochemistry: challenges and perspectives for therapeutic strategies in lung cancer patients. Virchows Arch 2016;468:511-25.
19. Le DT, Durham JN, Smith KN, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science 2017;357:409-13.
20. Rizvi H, Sanchez-Vega F, La K, et al. Molecular Determinants of Response to Anti-Programmed Cell Death (PD)-1 and Anti-Programmed Death-Ligand 1 (PD-L1) Blockade in Patients With Non-Small-Cell Lung Cancer Profiled With Targeted Next-Generation Sequencing. J Clin Oncol 2018;36:633-41.
21. Carbone DP, Reck M, Paz-Ares L, et al. First-Line Nivolumab in Stage IV or Recurrent Non-Small-Cell Lung Cancer. N Engl J Med 2017;376:2415-26.
22. Hellmann MD, Ciuleanu TE, Pluzanski A, et al.

Nivolumab plus Ipilimumab in Lung Cancer with a High Tumor Mutational Burden. N Engl J Med 2018;378:2093-104.
23. Hellmann MD, Nathanson T, Rizvi H, et al. Genomic Features of Response to Combination Immunotherapy in Patients with Advanced Non-Small-Cell Lung Cancer. Cancer Cell 2018;33:843-52.e4.
24. Socinski MA, Jotte RM, Cappuzzo F, et al. Atezolizumab for First-Line Treatment of Metastatic Nonsquamous NSCLC. N Engl J Med 2018;378:2288-301.
25. Legrand FA, Gandara DR, Mariathasan S, et al. Association of high tissue TMB and atezolizumab efficacy across multiple tumor types. J Clin Oncol 2018;36:abstr 12000.
26. Gajewski TF. Fast Forward - Neoadjuvant Cancer Immunotherapy. N Engl J Med 2018;378:2034-5.
27. Forde PM, Chaft JE, Pardoll DM. Neoadjuvant PD-1 Blockade in Resectable Lung Cancer. N Engl J Med 2018;379:e14.
28. Devarakonda S, Rotolo F, Tsao MS, et al. Tumor Mutation Burden as a Biomarker in Resected Non-Small-Cell Lung Cancer. J Clin Oncol 2018:JCO2018781963.
29. Ng SB, Turner EH, Robertson PD, et al. Targeted capture and massively parallel sequencing of 12 human exomes. Nature 2009;461:272-6.
30. Chalmers ZR, Connelly CF, Fabrizio D, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med 2017;9:34.
31. Horak P, Frohling S, Glimm H. Integrating next-generation sequencing into clinical oncology: strategies, promises and pitfalls. ESMO Open 2016;1:e000094.
32. Griffith M, Miller CA, Griffith OL, et al. Optimizing cancer genome sequencing and analysis. Cell Syst 2015;1:210-23.
33. Damodaran S, Berger MF, Roychowdhury S. Clinical tumor sequencing: opportunities and challenges for precision cancer medicine. Am Soc Clin Oncol Educ Book 2015:e175-82.
34. Hatakeyama K, Nagashima T, Urakami K, et al. Tumor mutational burden analysis of 2,000 Japanese cancer genomes using whole exome and targeted gene panel sequencing. Biomed Res 2018;39:159-67.
35. Frampton GM, Fichtenholtz A, Otto GA, et al. Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. Nat Biotechnol 2013;31:1023-31.
36. Fang P, Yan Z, Vu Q, et al. Evaluation of a commercial targeted NGS panel for tumor mutation burden assessment in FFPE tissue. Cancer Res 2018;78:abstr 3614.
37. Hofman P. Liquid Biopsy and Therapeutic Targets: Present and Future Issues in Thoracic Oncology. Cancers (Basel) 2017;9.
38. Khagi Y, Goodman AM, Daniels GA, et al. Hypermutated Circulating Tumor DNA: Correlation with Response to Checkpoint Inhibitor-Based Immunotherapy. Clin Cancer Res 2017;23:5729-36.
39. Fabrizio D, Lieber D, Malboeuf C, et al. A blood-based next-generation sequencing assay to determine tumor mutational burden (bTMB) is associated with benefit to an anti-PD-L1 inhibitor, atezolizumab. Cancer Res 2018;78:abstr 5706.
40. Gandara DR, Paul SM, Kowanetz M, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. Nat Med 2018;24:1441-8.
41. Newman AM, Bratman SV, To J, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20:548-54.
42. Hu Y, Ulrich BC, Supplee J, et al. False-Positive Plasma Genotyping Due to Clonal Hematopoiesis. Clin Cancer Res 2018;24:4437-43.
43. Ptashkin RN, Mandelker DL, Coombs CC, et al. Prevalence of Clonal Hematopoiesis Mutations in Tumor-Only Clinical Genomic Profiling of Solid Tumors. JAMA Oncol 2018;4:1589-93.
44. Do H, Dobrovic A. Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. Clin Chem 2015;61:64-71.
45. Buchhalter I, Rempel E, Endris V, et al. Size matters: Dissecting key parameters for panel-based tumor mutational burden (TMB) analysis. Int J Cancer 2019;144:848-58.
46. Garofalo A, Sholl L, Reardon B, et al. The impact of tumor profiling approaches and genomic data strategies for cancer precision medicine. Genome Med 2016;8:79.
47. Qiu P, Pang L, Arreaza G, et al. Data Interoperability of Whole Exome Sequencing (WES) Based Mutational Burden Estimates from Different Laboratories. Int J Mol Sci 2016;17.
48. VanderLaan PA, Rangachari D, Costa DB. Lung Cancer with a High Tumor Mutational Burden. N Engl J Med 2018;379:1093.
49. McGranahan N, Furness AJ, Rosenthal R, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 2016;351:1463-9.
50. Miao D, Margolis CA, Vokes NI, et al. Genomic correlates of response to immune checkpoint blockade

in microsatellite-stable solid tumors. Nat Genet 2018;50:1271-81.
51. Luksza M, Riaz N, Makarov V, et al. A neoantigen fitness model predicts tumour response to checkpoint blockade immunotherapy. Nature 2017;551:517-20.
52. Offin M, Rizvi H, Tenet M, et al. Tumor Mutation Burden and Efficacy of EGFR-Tyrosine Kinase Inhibitors in Patients with EGFR-Mutant Lung Cancers. Clin Cancer Res 2018. [Epub ahead of print].
53. Hellmann MD, Callahan MK, Awad MM, et al. Tumor Mutational Burden and Efficacy of Nivolumab Monotherapy and in Combination with Ipilimumab in Small-Cell Lung Cancer. Cancer Cell 2018;33:853-61.e4.
54. Boumber Y. Tumor mutational burden (TMB) as a biomarker of response to immunotherapy in small cell lung cancer. J Thorac Dis 2018;10:4689-93.

Cite this article as: Berland L, Heeke S, Humbert O, Macocco A, Long-Mira E, Lassalle S, Lespinet-Fabre V, Lalvée S, Bordone O, Cohen C, Leroy S, Hofman V, Hofman P, Ilié M. Current views on tumor mutational burden in patients with non-small cell lung cancer treated by immune checkpoint inhibitors. J Thorac Dis 2019;11(Suppl 1):S71-S80. doi: 10.21037/jtd.2018.11.102