REVIEW ARTICLES

The Promises and Challenges of Tumor Mutation Burden as an Immunotherapy Biomarker: A Perspective from the International Association for the Study of Lung Cancer Pathology Committee

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ABSTRACT

Immune checkpoint inhibitor (ICI) therapies have revolutionized the management of patients with NSCLC and have led to unprecedented improvements in response rates and survival in a subset of patients with this fatal disease. However, the available therapies work only for a minority of patients, are associated with substantial societal cost, and may lead to considerable immune-related adverse events. Therefore, patient selection must be optimized through the use of relevant biomarkers. Programmed death-ligand 1 protein expression by immunohistochemistry is widely used today for the selection of programmed cell death protein 1 inhibitor therapy in patients with NSCLC; however, this approach lacks robust sensitivity and specificity for predicting response. Tumor mutation burden (TMB), or the number of somatic mutations derived from next-generation sequencing techniques, has been widely explored as an alternative or complementary biomarker for response to ICIs. In theory, a higher TMB increases the probability of tumor neoantigen production and therefore, the likelihood of immune recognition and tumor cell killing. Although TMB alone is a simplistic surrogate of this complex interplay, it is a quantitative variable that can be relatively readily measured using currently available sequencing techniques.

A large number of clinical trials and retrospective analyses, employing both tumor and blood-based sequencing tools, have evaluated the performance of TMB as a predictive biomarker, and in many cases reveal a correlation between high TMB and ICI response rates and progression-free survival. Many challenges remain before the implementation of TMB as a biomarker in clinical practice. These include the following: (1) identification of therapies whose response is best informed by TMB status; (2) robust definition of a predictive TMB cut point; (3) acceptable sequencing panel size and design; and (4) the need for robust technical and informatic rigor to generate precise and accurate TMB measurements across different laboratories. Finally, effective prediction of response to ICI therapy will likely require integration of TMB with a host of other potential biomarkers, including tumor genomic driver alterations, tumor-immune milieu, and other features of the host immune system. This perspective piece will review the current clinical evidence for TMB as a biomarker and address the technical sequencing considerations and ongoing challenges in the use of TMB in routine practice.

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Keywords: TMB; NSCLC; Immunotherapy; Biomarker; PD-L1

Introduction

Immune checkpoint inhibitor (ICI) therapies, including those targeting the programmed cell death protein 1 (PD-1)–programmed death-ligand 1 (PD-L1) and CTLA4-CD80 (B7-1)–CD86 (B7-2) interactions, exploit tumor–immune-cell interactions to effect tumor cell killing. Therapeutic benefit from ICI is enriched in certain tumor types, including but not limited to melanoma, NSCLC, head and neck carcinomas, bladder carcinomas, renal cell carcinoma, and mismatch repair (MMR)–deficient/microsatellite instability–high carcinomas irrespective of the site of origin. As a result, the Food and Drug Administration (FDA), the European Medicines Agency (EMA), and other regulatory agencies across the globe have approved a variety of ICI-based therapies for these indications and more. In the lung cancer space, PD-1 and PD-L1 inhibitors are now the first-line standard of care in all "driver-negative" patients with metastatic and local, nonradically treatable advanced NSCLC and later-lines for relapsed/refractory extensive-stage SCLC.[1,2] As overall survival (OS) data mature for ICI trials, it is becoming evident that this therapeutic approach offers a consistent survival benefit over chemotherapy across cancer populations and provides long-term benefit in a subset of patients with metastasis, validating this strategy and ensuring that harnessing of the tumor-immune response will remain a central tenet of cancer therapy.[3,4]

Despite the great promise of ICI therapy, consistent clinicopathologic and genomic predictors of response to ICI remain elusive. Only a minority of NSCLC clearly benefit from ICI monotherapy, and we continue to struggle to accurately identify these patients.[5] PD-L1 protein expression, as evaluated by immunohistochemistry (IHC), has been assessed and established as a predictive biomarker for ICI in the context of the first NSCLC clinical trials, using several assays that have been compared, harmonized, and validated.[6,7] As a result, PD-L1 IHC is the only regulatory agency–approved biomarker for ICI selection for NSCLC patients.[8] PD-L1 IHC is formally required only in consideration for first-line pembrolizumab monotherapy in advanced NSCLC and with the use of consolidation durvalumab after chemoradiation for stage III locally advanced disease in Europe. These restricted indications for PD-L1 testing reflect its limitations as a biomarker, namely its insufficient negative-predictive value, its failure to consistently predict outcome benefits using immunotherapy-based combination therapies,[9-11] and its lack of established predictive role to date in SCLC and other cancer types. Other approaches to ICI therapy selection, therefore, are needed.

A high somatic mutation burden is a unifying feature of many of the cancer types for which ICI therapies have proven effective to date. Indeed, pancancer analyses reveal a statistical correlation between tumor mutation burden (TMB) and response rates and outcomes after PD-1 inhibitors.[12] Tumor mutations arise as a result of DNA damage from exogenous factors (tobacco smoke, ultraviolet light, DNA-damaging therapies) or as a result of defects in endogenous DNA repair pathways (MMR, homologous recombination, base excision repair). With the advent of low cost, high-throughput genome sequencing, we are now able to discern both quantitative and qualitative differences in mutational patterns across tumor types (Fig. 1).[13-15] From the standpoint of ICI efficacy, tumor mutations are possibly relevant when they generate novel mutated proteins or neoantigens, which may be recognized as foreign by the immune system and trigger a cytotoxic, tumor-killing response. In theory, tumors with a higher number of mutations are statistically more likely to generate neoantigens,[16,17] and certain mutation types, including the frameshift mutations common in MMR-deficient tumors, generate neoantigens in higher numbers and greater potency, likely explaining the striking efficacy of ICI in this setting.[18] Thus, in individual patients both TMB itself and the character of the underlying mutational changes may provide additional insight into the likelihood of an effective tumor–immune-cell interaction.[17]

Correlative and Clinical Trial Data Supporting the Use of Tissue TMB as a Biomarker in NSCLC

A summary of the clinical evidence supporting TMB's utility as a predictive biomarker for ICI therapy in patients with NSCLC is provided in Table 1.[19-29] In a large, single-institution study of patients receiving diverse ICI therapies across many tumor types, Samstein et al.[15] confirmed the predictive association of high TMB as derived from a targeted 468 cancer gene next-generation sequencing (NGS) panel (Memorial Sloan Kettering–Integrated Mutation Profiling of Actionable Cancer Targets [MSK-IMPACT]). In this large cohort, the authors found that TMB was significantly associated with OS, both as a continuous variable and with a binary cutoff (top 20% of each cancer type), using a multivariate approach adjusting for cancer type. The authors excluded the possibility that these results were confounded by a prognostic impact of TMB by evaluating a separate cohort of over 5000 ICI-naive patients sequenced by MSK-IMPACT and showing that high TMB offered no prognostic benefit.[15] For most cancer types, an association between higher TMB and improved survival was observed. However, TMB cutpoints associated with improved survival varied markedly between tumor types.

Table 1. Summary of Clinical Evidence Revealing TMB as a Biomarker for Response to Immunotherapy in Lung Cancer

Immunotherapy Agent	Study/Trial	TMB Assay Used	Type of Benefit
Nivolumab			
NSCLC (1L)	CheckMate 026[31]	WES	ORR, PFS
NSCLC	Flatiron Health[20]	Foundation CGP panel	OS
Nivolumab and ipilimumab combination			
NSCLC (1L)	CheckMate 012[49]	WES	ORR, DCB, PFS
NSCLC (1L)	CheckMate 227[33]	FoundationOne CDx	ORR, PFS
NSCLC (1L)	CheckMate 568[21]	FoundationOne CDx	ORR
SCLC (2L)	CheckMate 032[22]	WES	ORR, OS, PFS
Pembrolizumab			
NSCLC (1L)	Keynote-001[30]	WES	ORR, DCB, PFS
Atezolizumab			
NSCLC (2L)	POPLAR/OAK[23,38]	Foundation bTMB	OS, PFS
NSCLC (2L)	POPLAR/FIR/BIRCH[24]	FoundationOne	ORR, OS, PFS
NSCLC (1L)	BFAST and B-F1RST[25-27]	Foundation bTMB	DOR, ORR, PFS, OS
NSCLC (1L)	Rizvi et al., 2018[61]	WES	DCB, ORR, PFS
Durvalumab			
NSCLC (1L)	MYSTIC[28,37]	FoundationOne CDx	OS
Durvalumab and tremelimumab combination			
NSCLC (1L)	MYSTIC[28,37]	FoundationOne CDx	OS
NSCLC (1L)	MYSTIC[28,37]	Guardant OMNI bTMB	OS
Multiple Agents			
NSCLC	Rozenblum et al., 2017[29]	FoundationOne Guardant 360	ORR
NSCLC	Samstein et al., 2019[15]	MSK-IMPACT	OS

Adapted from Büttner et al.[19]
BFAST, Blood First Assay Screening Trial; B-F1RST, Blood First Line Ready Screening Tool; bTMB, blood tumor mutational burden; CGP, comprehensive genomic profiling; DCB, durable clinical benefit; DOR, duration of response; MSK-IMPACT, Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets; ORR, objective response rate; OS, overall survival; PFS, progression-free survival; TMB, tumor mutational burden; WES, whole-exome sequencing.

Rizvi et al.[30] published the first promising correlative biomarker data on the basis of two clinical trial cohorts of patients with NSCLC treated with pembrolizumab. This retrospective analysis revealed that a high non-synonymous mutation burden, defined as greater than the median number of mutations detected by whole-exome sequencing (WES), predicted significantly greater durable clinical benefit and progression-free survival (PFS).[30] In the CheckMate 026 study, a phase III study comparing the PD-1 inhibitor nivolumab to platinum doublet chemotherapy in the first-line setting for advanced NSCLC that express PD-L1 on greater than 5% of tumor cells, no statistical PFS difference was seen between the two treatment arms. However, a retrospective analysis of patients with high TMB (defined as harboring greater than 243 missense mutations by WES) had a prolonged PFS (median 9.4 versus 5.4 mo, hazard ratio [HR] = 0.62; 95% confidence interval [CI]: 0.38–1.00) and higher objective response rate (ORR) (46.8% versus 28.3%), but a nonsignificant OS difference.[31]

In CheckMate 568, a single-arm phase II study evaluating first-line treatment of advanced NSCLC with low-dose ipilimumab and nivolumab,[32] a protocol amendment was implemented after enrollment was completed to assess efficacy by TMB as a secondary end point and determine an appropriate TMB cutoff for further validation in the phase III CheckMate 227 trial. In the TMB-evaluable group (n = 98), ORR improved with increases in TMB to a plateau at TMB greater than or equal to 10 mutations per Mb. ORR was 44% in patients with TMB greater than or equal to 10 mutations per Mb versus 12% in patients with less than 10 mutations per Mb, with higher ORRs observed in those with high TMB, independent of PD-L1 expression level. PFS was prolonged in patients with greater than or equal to 10 mutations per Mb versus those with less than 10 mutations per Mb (7.1 mo versus 2.6 mo, respectively).

On the basis of CheckMate 568, a prespecified TMB cutoff of 10 mutations per Mb was selected for Check-Mate 227, which assessed PFS with low-dose ipilimumab plus nivolumab versus chemotherapy in patients with high TMB, irrespective of PD-L1 expression, as one of two coprimary end points.[33] In the high TMB population, the 1-year PFS was 42.6% with nivolumab plus ipilimumab versus 13.2% with chemotherapy, with a median PFS of 7.2 months versus 5.5 months, and an HR for disease progression or death of 0.58 (97.5% CI: 0.41–0.81). In contrast, the median PFS of the low TMB group

was 3.2 months (HR 1.07; 95% CI: 0.84–1.35 versus chemotherapy). Of concern in this study was the 42% failure rate in obtaining a TMB score despite the use of a targeted NGS panel; however, the late TMB end point addition for this trial might have resulted in an insufficient tissue requirement per protocol. Subsequent exploratory OS analysis found no significant difference in survival outcomes between high or low TMB groups, with HR for OS of 0.77 (95% CI: 0.5–1.06) for high TMB patients versus 0.78 (95% CI: 0.61–1.00) for low TMB. Median OS values (quoted in the initial press release[34] as 16.2 versus 12.4 mo in low TMB and 23.3 versus 16.7 mo in high TMB for ipilimumab/nivolumab versus chemotherapy, respectively) suggested a confounding prognostic impact of TMB with a notably high OS of almost 2 years in patients with high TMB treated with ipilimumab/nivolumab. This prognostic effect of TMB, specifically impacting the chemotherapy arm, was not reported in any other trial, except in the CheckMate 026 described above. The final report of OS for CheckMate 227, with more than 23 months of follow-up, revealed an improvement in OS, PFS, response rate, and duration of response for low-dose ipilimumab and nivolumab versus chemotherapy, in the dual coprimary end point of PD-L1–positive cohort but also irrespective of PD-L1 expression, TMB or any combination of the two.[35]

Retrospective analysis of WES-based TMB in the pembrolizumab monotherapy frontline KEYNOTE-042 and second-line KEYNOTE-10 randomized trials versus chemotherapy reinforced the available evidence regarding the predictive value of TMB for immunotherapy. In this exploratory analysis, higher TMB, as a continuous variable, was associated with improved clinical outcomes for pembrolizumab monotherapy in patients with PD-L1–positive advanced NSCLC. In general, TMB was not associated with response to chemotherapy treatment.[36] Of interest, the addition of platinum-based chemotherapy to pembrolizumab in the frontline randomized trials KEYNOTE-21, -189, and -407 versus chemotherapy cancelled the predictive ability of TMB. No association between TMB and efficacy for pembrolizumab and chemotherapy or chemotherapy and placebo could be observed. These data suggest that TMB may have limited clinical utility in the setting of a combination of ICI and chemotherapy for both metastatic squamous and nonsquamous NSCLC.

Clinical Trial Data Suggesting Use of Blood TMB as a Biomarker in NSCLC

Trials are evolving to incorporate blood-based biomarker testing, in particular the use of NGS-based panel tests optimized for mutation detection in cell-free DNA. In the MYSTIC trial, exploratory OS analysis

of tissue-based TMB at a cutoff of 10 mutations per Mb found improved median OS in patients with high TMB treated with durvalumab (18.6 mo) or a combination of durvalumab and tremelimumab (16.6 mo) versus chemotherapy alone (11.9 mo), whereas patients with low TMB did better with chemotherapy alone, although these differences did not achieve statistical significance.[37] However, tissue TMB (tTMB) was available in only 41% of the patients, a reflection of the challenges in tissue sample adequacy for biomarker testing. Data on blood TMB (bTMB) analysis for the MYSTIC study, on the basis of a 500-gene panel with a 1.0 Mb sequence output (Guardant OMNI, Guardant Health), has been presented by Peters et al.[37] and Rizvi et al.[28] The study data set included 809 samples comprising 72.4% of the intention-to-treat population and used a prespecified exploratory analysis of high bTMB defined as greater than or equal to 16 mutations per Mb cutoff. At this cutoff, high bTMB was associated with improved survival for combination therapy, with an OS of 16.5 months versus 10.5 months for chemotherapy (HR 0.62; 95% CI: 0.45–0.86), compared with an OS of 8.5 months for patients with low bTMB receiving combination therapy versus 11.5 months for chemotherapy.[28] tTMB (FoundationOne) and Guardant OMNI bTMB revealed a positive correlation with a Spearman's rho of 0.6 and Pearson's *r* of 0.7. Additional analyses revealed increasing OS benefit with higher bTMB cutoffs. For patients with bTMB greater than or equal to 20 mutations per Mb, median OS was 21.9 months for those receiving combination therapy versus 10.0 months for chemotherapy (HR 0.49, 95% CI: 0.32–0.74), compared with an OS of 8.5 months for patients with bTMB less than 20 mutations per Mb receiving combination therapy versus 11.6 months for chemotherapy.[37] Data from this study also support the hypothesis that bTMB and PD-L1 expression are independent biomarkers with only a 9% overlap between the bTMB greater than or equal to 20 mutations per Mb and PD-L1 greater than or equal to 25% tumor expression level when evaluated with the SP263 assay.

Gandara et al.[38] reported the utility of the bTMB approach in a subset of patients enrolled in the POPLAR and OAK clinical trials. Using a 1.1 Mb coding sequence targeted panel assay (Foundation Medicine, Cambridge, MA), the authors reported a positive correlation between TMB derived from the pretreatment plasma of 259 patients, and matched tissue tested by FoundationOne CDx (the Spearman rank correlation of 0.64 (95% CI: 0.56–0.71). Interestingly, in patients with high TMB (defined as >30 mutations/sample) in both tTMB and bTMB assays, one-third of variants detected were unique to the blood sample and a quarter were exclusive to the tissue sample. The authors suggested that heterogeneity across

tumor compartments might impact our interpretation of the tumor genome, stressing the fundamental technical differences in computational pipelines and sample characteristics between tTMB and bTMB.[39] Retrospective analysis of samples from the POPLAR study determined that a bTMB cut point of greater than or equal to 16 mutations/sample had the strongest PFS treatment effect and an overall prevalence of 30%. Similarly, data from the OAK study on the groups with bTMB greater than or equal to 16 revealed a significant PFS benefit (HR 0.65 95% CI: 0.47–0.92) from atezolizumab versus docetaxel.[38]

The B-F1RST study is the first prospective data set evaluating bTMB as a predictive biomarker for atezolizumab response in NSCLC. In this phase II study, ORR was 28.6% compared with 4.4% for high versus low bTMB, using a prespecified cutoff of 16 mutations/sample (bTMB score of 16 ≈ 14.5 mutations per Mb).[40] At the time of the final analysis (at least 18 months of follow up), PFS was 3.5 months in the TMB-low versus 5.0 months for the TMB-high category (HR 0.80; 90% CI: 0.54–1.18), and OS was 13.4 months in the TMB-low and 23.9 months in the TMB-high category (HR 0.66; 90% CI: 0.40–1.10). HR for PFS and OS improved between high and low bTMB groups with increasing bTMB thresholds, from HR 1.16 and 0.95 at a threshold of bTMB greater than or equal to 10 to HR 0.59 and 0.44, respectively, at a threshold of bTMB greater than or equal to 20.[41] Other prospective studies of bTMB as a predictive biomarker for ICI (including the basket Blood First Assay Screening Trial [BFAST] trial) are ongoing.[42]

TMB as a Predictive Biomarker in SCLC

SCLC is characterized by high TMB.[43] Nivolumab received FDA accelerated approval in August 2018 for third-line treatment of metastatic SCLC, irrespective of PD-L1 or TMB status, on the basis of subgroup analysis of patients with SCLC in the CheckMate-032 study.[44] More recently, FDA approval was granted for pembrolizumab for second-line treatment of metastatic SCLC, irrespective of PD-L1 or TMB status on the basis of the analysis of 83 patients with SCLC from the KEYNOTE-028 and KEYNOTE-158 trials.[45] Monotherapy with immunotherapy is not yet EMA-approved for SCLC. Emerging data suggest a potential role for TMB as a predictive biomarker in SCLC. Evaluation of TMB by WES in a subset of 211 patients with SCLC from CheckMate 032 revealed enhanced efficacy of nivolumab plus or minus ipilimumab in patients with high TMB scores in the upper tertile (≥248 somatic missense mutations per Mb), compared with patients in the midtertile (143–247 mutations per Mb) and the low-tertile (<143 mutations per Mb) TMB.[46] Increases in ORR, PFS, and OS were observed in patients in the upper tertile TMB compared

with patients with mid- or low-tertile scores, with more pronounced effects observed for the combination of nivolumab plus ipilimumab versus nivolumab alone. Estimated 1-year OS for nivolumab plus ipilimumab was significantly higher in the high TMB group (62.4%) compared with the mid- and low-tertile TMB groups (19.6% and 23.4%, respectively), with a median OS of 22.0 months in the high TMB group (95% CI: 8.2 mo to not reached) compared with 3.6 months (95% CI: 1.8–7.7 mo) and 3.4 months (95% CI: 2.8–7.3 mo) for the mid- and low-tertile TMB groups, respectively.[47] Although the addition of atezolizumab frontline to platinum-based chemotherapy was reported to improve OS as compared with classical chemotherapy in extensive-stage SCLC in the IMPower 133 clinical trial, bTMB did not reveal any predictive ability for this chemotherapy-containing combination, similar to the NSCLC experience.[48]

Technical Considerations in TMB Analysis

WES As Definitive Standard for TMB Analysis

TMB denotes the number of acquired somatic mutations in the coding region of the cancer cell genome—that is, all exons of the approximately 22,000 human genes. Although whole-genome sequencing should represent the ground truth for reasons of cost and ease of analysis, WES is generally considered as the definitive standard for TMB estimation. The full exon backbone amounts to 30 to 35 Megabases (Mb), depending on the capture protocol used. As a proxy for neoantigen load, usually only non-synonymous point mutations that cause amino acid substitutions in the resulting proteins are included in the TMB count.[30] Splice site and small insertion-deletion (indel) mutations may be included variably in calculations of TMB, and some investigators have begun investigating whether the inclusion of synonymous variants—although these are unlikely to contribute substantially to neoantigen production—may improve the predictive performance of TMB.[49] As larger structural genetic aberrations (e.g., gene rearrangements) are difficult to assess and quantify in a standardized fashion by DNA-based targeted or exome NGS methods, and their impact on immunotherapy response remains obscure (see below—other immune-oncology [IO] biomarkers), they are not currently considered in the definition of TMB. Similarly, although certain amplification events involving gene encoding checkpoint proteins (*CD274, PDCD1LG2*) are potent predictors of ICI activity in certain tumor types (Hodgkin's lymphoma, mediastinal large B cell lymphoma),[50,51] to date the profile of genomic copy gains and losses has not been incorporated into the concept of TMB.

The number of nonsynonymous mutations in a tumor exome is conceptually a straightforward biomarker for

molecular pathology. However, the large size of a WES panel requires extensive sequencing capacity, larger DNA input, and larger tumor samples than most current clinical methodologies, and ideally, parallel sequencing of a normal, matched sample for removal of germline variants. During the past decade, WES has primarily been used as an exploratory research tool for tumor profiling on unfixed fresh-frozen tissue, prototypically in The Cancer Genome Atlas (TCGA) and other cancer atlas projects and has only recently matured for use on formalin-fixed, paraffin-embedded (FFPE) specimens with regard to sequencing chemistry and dedicated bioinformatic pipelines for handling formalin-induced sequencing artifacts.[52] Moreover, owing to the moderate standard sequencing depth (approximately ×100–250) of WES, subclonal mutations with low mutant allele frequency can be harder to detect. Thus, WES demands samples with high DNA quality and high tumor cell fraction and excludes routine samples with low quality and low tumor cell content typically encountered in lung cancer diagnostics. These technical challenges with additional logistical challenges have prompted the development of smaller, more focused NGS panels in clinical practice.

From WES to large NGS panels—Comprehensive Genomic Profiling

Comprehensive genomic profiling of a patient's cancer tissue sample (by use of large, focused NGS panels of 300–800 genes) aims to identify targetable genetic aberrations in the form of point mutations and single nucleotide variants, indels, amplifications, deletions, and gene rearrangements and mutation patterns such as microsatellite instability status and TMB.[53] Although most of these aberrations can be detected by DNA-based NGS panels, gene rearrangements require extra care in the design of the capture probes owing to breakpoints being distributed in vast intronic regions that might harbor repetitive sequences that can elude capture. Even with optimal capture design, targeted DNA-based panels can miss fusion events, warranting the addition of RNA-based methods.[54] For TMB estimation using such panels, several parameters influence the performance of the assay and might explain discordant results between different analysis platforms. How these parameters are addressed in representative targeted DNA-based panels is illustrated in Table 2.[55–57]

Panel Size. In silico analyses of WES data have revealed that panels of approximately 300 plus genes (approximately 1 Mb[58]) or above 1.5 Mb[59] can deliver TMB estimations with good concordance to WES, even in the low to moderate stratum of TMB counts. Empirical head-to-head comparisons between WES and established panels from Foundation Medicine (FoundationOne CDx, 0.8 Mb[60]) and Memorial Sloan-Kettering (MSK-IMPACT, 0.98–1.22 Mb[61]) support these data.

The Type of Aberration to Count. Although nonsynonymous/missense mutations are the defining, potentially immunogenic aberrations for reported TMB

Table 2. Examples of Targeted DNA-Based Assays That Generate a TMB Score

Assay	Total size/ Coding (Mb)	Aberration in Algorithm[a]	Germline Filtering	Cancer Gene Bias Correction	FFPE Error Correction	Targeting	Reference
MSK-IMPACT	1.5/1.14	SNV (NS), indels	Paired normal	No	Pool of normals	Hybrid capture	15
FoundationOne CDx	2.2/0.8	SNV (NS, S), indels	Database, SGZ	Yes	Bioinformatic	Hybrid capture	55
Illumina TSO500	1.9/1.3	SNV (NS, S), indels	Database, SGZ	Yes	UMI	Hybrid capture	59
Thermo Fisher Oncomine	1.7/1.2	SNV (NS)	Database	No	UDG; deamination metric	Amplicon	56
Qiagen QIAseq TMB	1.3/1.3	SNV (NS), indels	Database	No	UMI	Amplicon	Personal communication, Raed Samara, Qiagen
NEO New Oncology, NEOplus ROU	2.5/1.2	SNV (NS), indels	Database	NA	Bioinformatic	Hybrid capture	76
Caris SureSelect XT	1.6/1.4	SNV (NS), indels	Database	No	Bioinformatic	Hybrid capture	57

[a]Aberrations included in the bioinformatic pipeline to estimate TMB. All assays report TMB according to the definition—that is, NS-TMB per Mb correlated to WGS.

FFPE, formalin-fixed paraffin-embedded; indel, insertion-deletion; MSK-IMPACT, Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets; NA, not available; NS, nonsynonymous; S, synonymous; SGZ, somatic-germline zygosity algorithm; SNV, single nucleotide variants; TMB, tumor mutational burden; UDG, uracil-DNA glycosylase; UMI unique molecular identifier.

values, other types of mutations can be included in the TMB algorithm—that is, indels, synonymous mutations, splice sites, and other intronic mutations; and perhaps even mutations occurring outside the coding regions. The rationale for their inclusion is obtaining additional events to count for increasing the resolution of the TMB estimation in smaller gene panels. Although most of the added noncoding mutations do not affect the immunogenicity of the tumor, they serve as proxies for the overall mutational rate of the tumor. More importantly, this strategy requires assay and cancer-specific correction factors to shift the resulting count into an extrapolated "nonsynonymous" TMB value for the clinical report.[58] Indel burden, in particular, may be informative in high-TMB cases.[39]

Need for Normal Matched Sample. Sequencing of paired tumor-normal matched samples with germline variants removed from the TMB count is regarded as the definitive standard for TMB analysis.[30] However, most assays aiming at diagnostic implementation use public databases to remove known germline polymorphisms to create a list of likely somatic mutations. A concern with this approach is the possibility of ethnicity bias in available germline variant databases, resulting in potentially "false-positive" somatic mutation calls in populations that are poorly represented in current databases.[58] An additional in silico strategy that can be used in a tumor sample consisting of a mix of cancer and normal cells is on the basis of differences in allele distribution and frequencies between somatic and germline events owing to copy number aberrations in a given chromosomal region in the cancer cell compartment (somatic-germline zygosity algorithm[62]). Furthermore, the presence of clonal hematopoiesis (CH)–derived mutations in the tumor specimen, owing to blood infiltration, necessitates the use of a matched peripheral blood sample to identify and remove these variants in an unbiased manner.[63]

Cancer Gene Bias. NGS panels focused on somatic cancer mutations are selectively biased to cover genes with recurrent mutations. Depending on the size of the panel and included genes, this could lead to an overestimation of TMB in the analyzed coding region. To reduce cancer gene bias, some platforms subtract recurrent cancer mutations (present in the Catalogue of Somatic Mutations in Cancer database) from the TMB count.[60]

Artifacts in FFPE. Formalin chemically induces DNA base damage, prototypically the deamination of cytosine (C) residues. During polymerase chain reaction-amplification, the polymerase erroneously incorporates an adenine (A) across the deaminated C instead of thymine (T), and after further amplification the C:G base

pair is replaced by a T:A base pair, resulting in the so called "C>T" artifact.[64] These artifacts usually occur at a low allele frequency and can be filtered as noise by a reasonable variant-calling cutoff (usually 5% allele frequency). Unfortunately, C>T artifacts occur at high frequencies in some samples, which might create false-positive driver mutations and lead to overestimation of TMB. Sequencing data from a cohort of FFPE-processed normal tissues can help identify the false-positive variants by investigating the recurrence of each called mutation in the pool of normal cohort. As base damage typically only affects one of the two complementary strands in captured DNA-fragments, artifacts can be filtered specifically by strategies that track discordant bases originating from a single double-stranded DNA-fragment,[65] for example, by the help of unique molecular identifiers. Another strategy is to reduce/reverse formalin-induced base damage by treating the DNA with uracil-DNA glycosylase, an enzyme that selectively digests uracil-containing nucleic acids.[65-68]

bTMB-Preanalytic and Analytical Considerations

Blood-based analysis of circulating tumor DNA (ctDNA) has been a welcome addition to FFPE-based biomarker testing in patients with NSCLC. ctDNA is present in human plasma as a result of apoptosis and cellular degradation.[69] The resulting ctDNA is highly fragmented and has a short half-life in peripheral circulation.[70] Studies have reported that at least 80% of patients with advanced NSCLC have detectable ctDNA in their bloodstream.[71] Careful preanalytic processes such as ctDNA preservative tubes or immediate separation of the plasma from other blood components are needed to avoid continued sample degradation after collection. Despite the challenges of working with ctDNA in a clinical laboratory, its clinical utility as a template for blood-based TMB testing has been evaluated in multiple early-stage clinical trials (as cited above), in which high bTMB has been associated with immunotherapy response.

When the number of mutations per megabase of DNA is compared between FFPE tissue and blood-based TMB analysis, there are notable differences between the values associated with TMB-high results. For the POPLAR and OAK studies, a tTMB level of greater than or equal to 10 mutations per Mb correlated with a bTMB value of greater than or equal to 16 mutations per Mb. There are several possible explanations for the differences, including bioinformatic calculations, tumor heterogeneity, ctDNA shedding, allelic fractions, assay performance, and patient stage at the time of sample collection.[38] The use of different laboratory tests for tissue and blood-based analysis may also contribute to the higher number of mutations detected in ctDNA-based

tests. As discussed above, Gandara et al.[38] suggested that the heterogeneity between a single tissue biopsy and ctDNA fragments in the blood released from multiple tumor sites may account for much of the difference; however, subsequent high-intensity sequencing studies of paired ctDNA and white blood cell fractions have revealed that DNA variants derived from CH in myeloid cells also make a substantial contribution to the presumed somatic alterations found in plasma-based testing.[72] This observation argues for paired sequencing of plasma and white blood cells to correct for CH, which is likely to be a confounder of bTMB studies, particularly in older populations in which CH is highly prevalent.[72,73]

TMB Assay Cross Comparison and Harmonization Efforts

Critical analysis of TMB as a biomarker has been complicated by the use of varied assay platforms across clinical trials and different cutoffs for the definition of high TMB. Technical issues contributing to variation across platforms include panel size, gene content, type of mutation included in the TMB count, access to a paired normal sample, and depth of sequencing. In general, the TMB derived from panel sequencing of several hundred genes (e.g., FMI, MSK-IMPACT) is comparable with WES; however, panel results consistently overestimate WES TMB as a result of their enrichment with genes affected in cancer and increased sensitivity to low-level tumor variants attributed to their increased depth of sequencing and optimized uniformity of coverage.[74] Tumor-only panels overestimate TMB as a result of "contaminating" germline variants, as discussed above, or as a result of variants derived from other subclinical disease processes such as CH.[63] However, it is possible to ascertain the degree to which an individual panel test overestimates a WES TMB result. Vokes et al.[75] have proposed the use of normal transformation followed by z score standardization to harmonize TMB scores derived from diverse assays. International harmonization efforts led by Friends of Cancer Research (FOCR) and Qualitätssicherungs-Initiative Pathologie are similarly endeavoring to quantify differences in TMB across platforms through cross-institutional testing and data sharing.[76,77] The FOCR TMB Harmonization Project is a three-phase effort, beginning with an in silico cross-comparison of TMB derived from TCGA mutation calling results by downsampling to a gene list of a given targeted panel. This in silico approach permitted relatively rapid and cost-effective analysis of a large number of samples (over 4000 individual tumors in each of the training and validation cohorts), all of which had previously undergone WES through TCGA. A total of 11

participating laboratories performed downsampling analysis on the basis of the panel of genes defined in their individual assays and using their unique pipeline thresholds. Across the entire data set, the Pearson correlation coefficient value for individual laboratory TMB and WES ranged from 0.85 to 0.93, with slopes ranging from 0.82 to 1.37. Notably, although these correlations appeared to be robust, variation was apparent between laboratories and was attributed to panel size, unique gene content, and inclusion (by some laboratories) of silent mutations. The correlation between TMB calculated from WES and panel sequence also varied according to tumor type, consistent with analyses done by Samstein et al.,[15] who reported that different TMB cutoffs predicted benefit after ICI in different tumor types. To capture other technical sources of variation, phase II of FOCR harmonization efforts will evaluate results after the sequencing of reference cell line samples within participating laboratories. Phase III will involve local sequencing of clinical samples to hone the standards required to allow for cross-laboratory harmonization for clinically relevant sample substrates. Analyses for phases II and III are ongoing at this time.[78]

Interrelatedness of TMB and Other IO/Genomic Biomarkers

A recent pantumor meta-analysis has revealed that multiplex IHC/immunofluorescence assays or multiparametric biomarker strategies are linked to better performance (i.e., higher area under the curve) over PD-L1 IHC, TMB, or transcriptional signatures alone.[79] To address the possibility that the real-world predictive value of these combined approaches is eventually confirmed, it is worth reviewing the interrelatedness of TMB and other biomarkers for checkpoint inhibitor immunotherapy (Fig. 1).

Although theoretically, PD-L1 expression is required to benefit from anti–PD-L1 or anti–PD-1 therapy, it is clear that it is an imperfect biomarker.[80] PD-L1 and TMB are independent biomarkers of response to both monotherapy and combination therapy.[49,61] Several observations derived from clinical trial data raise the possibility that TMB can identify a subgroup of PD-L1 non-expressers or low-expressers that may specifically benefit from ICI treatment strategies. Indeed, several studies have reported that the high-TMB and high–PD-L1 patient populations have limited overlap,[38,61] which could favor a complementary role for the two assays. The reliability of ICI response prediction improves when PD-L1 and TMB are combined in a multivariable model.[49,61]

Some driver genomic alterations can promote tumor neoantigens.[17,81] The predictive impact of *KRAS*

SMOKING
VIRAL INFECTION/INFLAMMATION/APOBEC
AGING

T
C/T
A

G
G/T
T

TUMOR MUTATIONS

Germline polymorphisms
Sequencing artifacts
Cytosine deamination

Tumor tissue

ctDNA

Sequencing

Paired normal variant subtraction
In silico germline filtering
Error correction/molecular barcoding

PD-L1

TMB
Total mutations (WES)
or
Mutations/Mb
(panel NGS)

Cancer Drivers
&
Co-mutations
KRAS
STK11
KEAP1
SMARCA4
EGFR
ALK

Immune
microenvironment

T-cell inflamed GEP

LAG3
IDO1
CYTOKINES
PD-L1/PD-L2
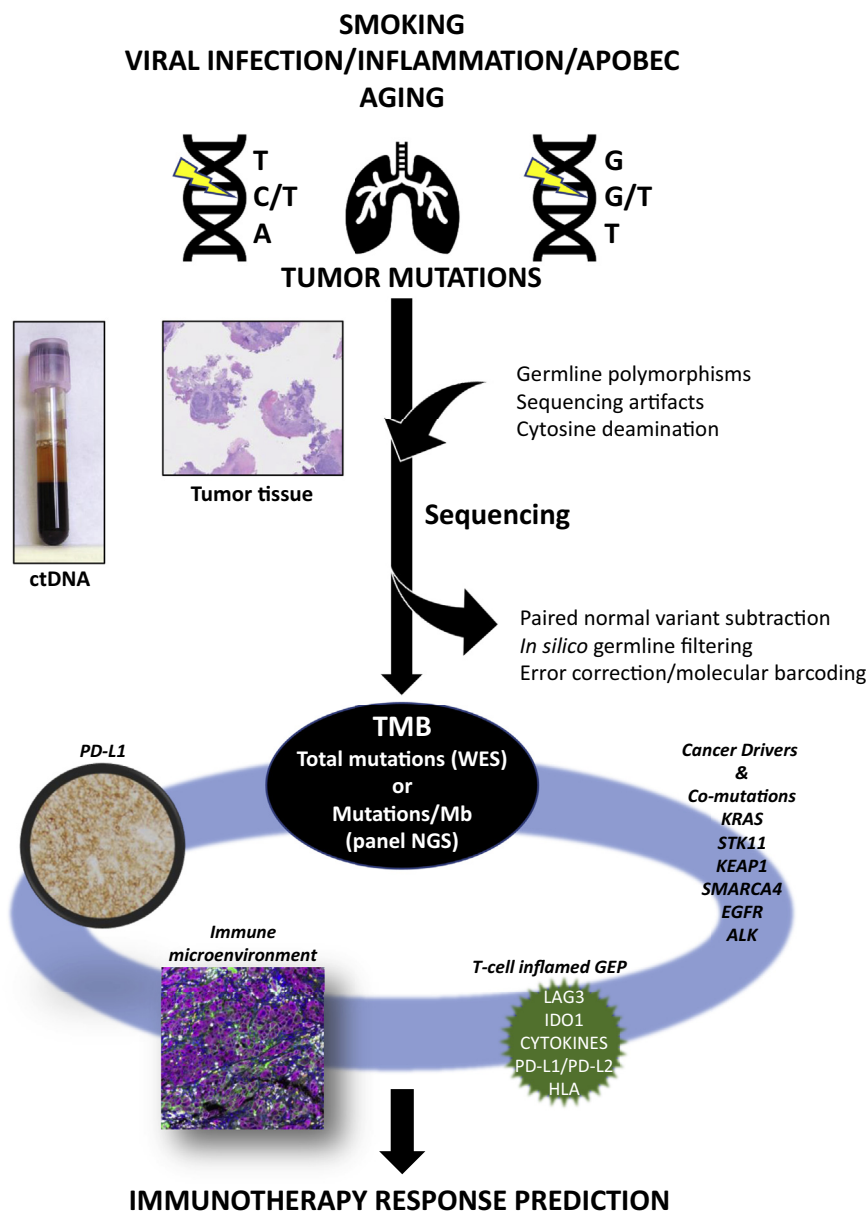HLA

IMMUNOTHERAPY RESPONSE PREDICTION

Figure 1. Exogenous exposures and endogenous DNA repair defects lead to an accumulation of mutations that may give rise to tumors in the lung. Tumor mutations can be detected and quantified in both blood and tumor tissue through NGS techniques. A subset of DNA variants represents germline polymorphisms or technical artifacts, which can be subtracted through the use of paired-tumor normal sequencing or bioinformatic approaches. The number of mutations detected is represented as tumor mutation burden—one of the many variables that may influence response to immune checkpoint blockade. ctDNA, circulating tumor DNA; GEP, gene expression profiling; NGS, next-generation sequencing; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; TMB, tumor mutation burden; WES, whole-exome sequencing.

mutations on response to ICI depends on the presence of concurrent mutations, despite similar median TMB values in three genomically defined subgroups (*KRAS*-only, *KRAS* + *LKB1,* and *KRAS* + TP53).[82] The best responses are seen when *TP53* is also present, whereas *KRAS+LKB1* comutations in lung adenocarcinomas are associated with primary resistance.[82] Irrespective of *KRAS* status, *LKB1* mutant tumors are enriched in PD-L1 negative and TMB intermediate/high subgroup,

emphasizing the need for comprehensive somatic profiling beyond TMB.[82] Several lines of evidence suggest that *EGFR* mutations are a reliable predictor of resistance to checkpoint blockade,[61,83,84] but a recent report suggests that outcomes are worse only in lung tumors with *EGFR* exon 19 alterations.[85] In agreement with the response data, adenocarcinomas with this latter genotype contained a lower TMB compared with exon 21 *EGFR*-mutant lung tumors.[85,86]

Other potential predictors of response are the density, location, and characteristics of the tumor-infiltrating lymphocytes. Multiplex IHC and immunofluorescence seem to be the most promising strategies for the assessment of the tumor–immune-cell environment and may indeed outperform other biomarkers and combinatorial biomarker strategies.[79] The additive benefit of TMB to a multiplex imaging modality in predicting ICI responses is unclear. In patients with NSCLC treated with pembrolizumab, no significant correlation has been found between TMB and CD8 lymphocytes measured with IHC.[87] Regarding the association of TMB and a T-cell–inflamed gene expression profile, the data generated with pantumor cohorts of patients after PD-1 axis blockade is controversial.[88,89] Despite a low correlation, the highest likelihood of response was seen in patients with high TMB and high expression of an 18-gene signature.[88,89]

The T-cell killing of cancer cells needs the antigen-presenting role of *HLA-I*. Accordingly, it has been hypothesized that zygosity in those genes affects the survival of patients treated with immunotherapy.[90] The additive effect of *HLA* class I heterozygosity and TMB seems to be driven by patients with melanoma in pancancer cohorts that also included NSCLC; patients with NSCLC included in this analysis were not enough to derive conclusions on the effect in this tumor type.[91] In a recent study on 646 NSCLC from three independent cohorts, the *HLA-I* genotype did not correlate with the outcome after ICI treatment.[92] An alternative explanation is that other mechanisms are more relevant in lung cancer (e.g., decreased expression of *HLA* class I).[91]

A T-cell exposed to a specific antigen is activated and clonally expands. The sequencing of the unique T-cell receptor (TCR) allows for the quantification of the T-cell clonal expansion.[93] T-cell clonality and restricted TCR-repertoire in the blood and tumor tissue are emerging as new predictive biomarkers, as revealed in melanoma.[94] In NSCLC treated with ICI, emerging data also suggest an association of higher intratumoral TCR clonality with better outcome at a metastatic stage and a reduced percentage of residual tumor in a neo-adjuvant PD-1 setting.[95,96] TCR clonality and diversity in the peripheral blood of patients with NSCLC may also serve as noninvasive predictors of response to ICI.[96,97] A higher TCR diversity index in *EGFR*-mutated than EGFR wild-type tumors might suggest a higher T-cell clonal expansion in EGFR wild-type tumors.[98] This could indirectly point to a possible reason for the unfavorable response of *EGFR*-mutated NSCLC to ICI. However, there are currently no published data on the predictive role of TCR clonality and diversity and their correlation with other biomarkers, including PD-L1 and TMB.

Epigenetic profiling has recently been proposed as another promising ICI biomarker with added value. In this retrospective study, a specific epigenetic signature called Epimmune predicted an improved outcome after PD-1 blockade, independent of PD-L1 status and TMB, whereas it had no prognostic effect in patients not treated by immunotherapy.[99]

Current Status and Controversies

TMB harbors a consistent predictive ability for immunotherapy, with convincing data in lung cancer, and promising similarities in several other solid tumors. However, to date, TMB has not been formally prospectively validated in the context of a prospective randomized trial. Most studies do not suggest any prognostic impact of TMB;[15] only a small number of them do, limiting any application of this biomarker as a predictive guide in treatment decisions until more data become available. Furthermore, TMB still lacks a satisfactory and reproducible definition and methodology.

In the absence of an approved indication for immunotherapy on the basis of TMB, when and how should information about this biomarker influence therapeutic decisions? The European Society for Medical Oncology clinical practice guidelines have proposed the use of TMB to select patients with NSCLC for first-line nivolumab and ipilimumab therapy on the basis of the coprimary PFS end point improvement in CheckMate-227. However, this combination has not been approved by regulatory agencies in this setting.[2] The recent observation of an OS benefit in a larger group of patients irrespective of TMB is likely to slow the implementation of TMB across centers. After the promising MYSTIC bTMB exploratory trial, the bTMB-dedicated, redesigned NEPTUNE trial evaluating durvalumab/tremelimumab/chemotherapy versus chemotherapy was announced as a negative trial in a press release,[100] questioning the power of this trial and again failing to establish the evidence for TMB use as a biomarker.

Despite the current requirement of only 1% PD-L1 tumor proportion score to select patients for first-line pembrolizumab monotherapy in the United States, only a small subset of patients whose tumors are low-positive for PD-L1 experience long-term benefit with this regimen.[3] As a result, clinicians usually favor the use of combined chemotherapy and pembrolizumab, rather than monotherapy, for tumors with PD-L1 tumor proportion score below 50%. This is also the indication for which pembrolizumab monotherapy is still registered in many regions, including EMA. An outstanding question is whether a high TMB score may help select patients with lower PD-L1 expression and across all PD-L1 strata with a greater likelihood of response to pembrolizumab

monotherapy. Low TMB scores might also identify patients with more than 50% PD-L1 who might also need chemotherapy. A convergence of thoroughly validated biomarkers including PD-L1 and TMB might help identify patients who would not benefit from currently available immunotherapies, which might be of paramount importance for the sustainability of many health systems in the future. Unfortunately, such biomarker-guided refined trials will most probably have to be led by large academic and international consortiums. These questions do not positively influence the drug market of the most important pharmaceutical companies and are unlikely to represent an industry priority in the coming years.

TMB derived from paired-tumor normal WES data from patients enrolled in KEYNOTE-021, KEYNOTE-189, and KEYNOTE-407 failed to reveal any association with response to pembrolizumab plus chemotherapy as compared with chemotherapy alone.[47] Owing to the lack of any differential signal, the first hypothesis would be the obliteration of the predictive ability in the context of the superimposed cytotoxic effect, independent of TMB, as for PD-L1 and potentially all immunotherapy biomarkers. This is further proven by the striking predictive ability of WES TMB using the same cutoffs, and of PD-L1 expression in KEYNOTE-42/10.[36] Of note, the selected TMB cut points in these KEYNOTE trial retrospective analyses (175 and 150 mutations per exome, correlating with 13 and 10 mutations per Mb by FMI) fall below those proposed by Samstein et al.[15] but are in the range of other studies, suggesting TMB's lack of predictive ability to date in NSCLC and further emphasizing the uncertainty about the cutoff to use.

Pivotal investigations of the cancer genome have uncovered oncogenic mutations and tumor suppressor inactivation underlying selective growth advantage, bringing into focus genomic changes as a measure of responsiveness to emerging targeted treatments. The same effort should be pursued beyond the count of mutation for TMB, adding granularity to the large gene sequencing effort in correlation to clinical responses to ICI. Analyzing the current database to decipher the evidence of the predictive impact of specific DNA repair gene alterations (MMR, polymerase epsilon, *BRCA1/2*), specific oncogenes (*KRAS, EGFR, ALK, BRAF, MET, MYC,* and others), tumor suppressors (*LKB1, KEAP1, TP53, PTEN*) and specific signaling pathways (such as tumor cell–intrinsic Wnt–β-catenin and SWI/SNF) will be crucial to improve and refine our reading of TMB in the future.

Despite this, large scale analyses of immunotherapy outcome data continue to suggest that PD-L1, TMB, and CD8+ tumor infiltration, and specific gene expression signatures can predict improved outcomes after immunotherapy. For TMB, we need to determine whether any cutpoint will be predictive in individual patients, whether this assessment would be feasible in daily practice focusing on blood-based analyses, and whether this should indeed influence the choice of therapy. More sophisticated approaches will most probably be required to leverage the information in the tumor genome for clinical decision making. Refining neoantigen prediction algorithms for neoepitopes may ultimately improve the ability to identify patients who are most likely to respond to checkpoint blockade. On its own, pretherapy TMB currently can help inform treatment decisions but does not provide unambiguous sensitivity or specificity in the treatment decision-making process.

Conclusions

In summary, TMB estimates the mutational load in the tumor genome and serves as a surrogate marker for tumor neoantigen production and potential immunogenicity. It is a continuous variable, and higher TMBs tend to correlate with improved outcomes after immune checkpoint therapy across tumor types. In addition, TMB and PD-L1 seem biologically interrelated but are independent biomarkers of response to immune checkpoint therapies for NSCLC.

No single tumor tTMB threshold has been shown to consistently predict improved progression-free and OS in prospective randomized control trials of immune checkpoint therapies. bTMB (derived from cell-free DNA sequencing studies) correlates with tTMB, but different thresholds define a "high" result. bTMB is a promising biomarker for predicting response to immune checkpoint blockade but has not yet been proven in phase III randomized controlled trials. Targeted sequencing–based TMB correlates with WES TMB; however, estimates vary between laboratories as a function of panel size, composition, and bioinformatic techniques. Efforts to harmonize panel sequencing for TMB estimation are ongoing.

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