Review Article

Implementing tumor mutational burden (TMB) analysis in routine diagnostics—a primer for molecular pathologists and clinicians

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Abstract: Tumor mutational burden (TMB) is a new biomarker for prediction of response to PD-(L)1 treatment. Comprehensive sequencing approaches (i.e., whole exome and whole genome sequencing) are ideally suited to measure TMB directly. However, as their applicability in routine diagnostics is currently limited by high costs, long turnaround times and poor availability of fresh tissue, targeted next-generation sequencing (NGS) of formalin-fixed and paraffin-embedded (FFPE) samples appears to be a more feasible and straight-forward approach for TMB approximation, which can be seamlessly integrated in already existing diagnostic workflows and pipelines. In this work, we provide an overview of the clinical implications of TMB testing and highlight key parameters including pre-analysis, analysis and post-analytical steps that influence and shape TMB approximation by panel sequencing. Collectively, the data will not only serve as a field guide and state of the art knowledge source for molecular pathologists who consider implementation of TMB measurement in their lab, but also enable clinicians in understanding the specific parameters influencing TMB test results and reporting.

Keywords: Tumor mutational burden (TMB); mutational load; panel; next-generation sequencing (NGS); sequencing

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Introduction

Tumor mutational burden (TMB) is emerging as new predictive biomarker to select patients that benefit from immune checkpoint inhibitor therapy (1-7). It is commonly defined as the total number of somatic coding mutations and associated with the emergence of neoantigens that trigger anti-tumor immunity (8-10). As a defense mechanism, tumors acquire expression of checkpoint regulators, like programmed death-ligand 1 (PD-L1), the action of which can be overcome in clinical practice with therapeutic antibodies against PD-1 (programmed cell death protein 1) or PD-L1 alone or in combination with CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) inhibitors or chemotherapy or more recently small-molecule kinase inhibitors (11-13). In numerous clinical trials over the

past few years (14-21), these therapies have demonstrated impressive anti-tumor activity and are already approved for a large number of tumor entities in various indications (22) including non-small cell lung cancer (NSCLC) (23,24). Up to now, patient selection for immuno-oncologic (IO) treatment has been mainly based on expression of PD-L1 as assessed by immunohistochemistry (25,26). However, accumulating data suggest a much greater utility of TMB for this purpose.

A retrospective analysis of the CheckMate (CM) 026 trial investigating first-line nivolumab *vs.* platinum-based chemotherapy in stage IV or recurrent NSCLC (27) uncovered that patients with high TMB showed higher response rates and longer progression-free survival (PFS) with PD-1 blockade (*Table 1*). Moreover, among TMB-high patients, a high (≥50%) PD-L1 expression distinguished an even more favorable subgroup with presumably "hot", immunologically reactive tumors, but it had no predictive value among TMB-low cases. In keeping with these data, a post-hoc analysis in the nivolumab-ipilimumab arm of the CM 012 trial (28) also showed longer PFS of TMB-high cases under IO therapy, especially if PD-L1 was additionally present, but limited benefit in the TMB-low subgroup regardless of PD-L1 expression. Conversely, by focusing on PD-L1 negative patients, the CM 227 trial demonstrated a significant and incremental benefit from administration of nivolumab alone or in combination with ipilimumab for TMB-high cases, while additional presence of low TMB defined a truly refractory subset, for which novel IO approaches or non-IO treatment will be necessary (31,40). Notably, in all aforementioned analyses, PD-L1 expression was not associated with TMB levels, indicating that the two biomarkers are largely independent in NSCLC and probably also in other cancers (41). In addition, their results collectively may suggest a more fundamental role of a higher TMB for efficacy of currently available IO treatments, which therefore emerges as a potentially more suitable basis for the selection of NSCLC patients likely to benefit from them. At present, caution is definitely warranted, because overall survival (OS) data in conjunction with TMB are still pending and TMB measurement has not been used yet as an upfront stratification parameter in a prospective trial design. However, recent preliminary data suggest that higher TMB, but not higher PD-L1 expression levels, can reliably distinguish NSCLC patients predestined to experience long-term (>18 months) remissions with IO treatments, which is of utmost clinical importance, because it represents a proxy for "functional cure" (42). Since TMB appears to be predictive for efficacy of checkpoint blockade across diverse cancers (2,3), it is as an additional biomarker next to PD-L1 expected to improve care of many patients, which currently drives intense efforts for seamless integration of TMB assays in routine diagnostics.

TMB and cutoff values

Similar to the challenges of using PD-(L)1 expression as a biomarker for patient selection (43), there are many unsolved aspects of TMB assessment and interpretation which will need to be addressed before widespread adoption. Mutational burden is a continuous variable and the question arises how to define TMB-high tumors to enrich for patients likely to respond to IO therapy. Objective cut-points for TMB are not universally established. Clinical trials have mostly deferred study-specific cut-points using median TMB or dividing patients in tertiles or quartiles according to measured TMB (*Table 1*). Studies of NSCLC employing whole exome sequencing (WES) have for example set cut-points at 158 (median) (28), 200 (median) (6), and 243 (upper tertile) (27) mutations per exome. A study of SCLC determined 248 mutations per exome (34) to delineate the upper tertile, which is largely comparable to NSCLC. In a study of urothelial carcinoma, however, a lower number of 167 total mutations (35) was found to mark the upper tertile of patients. WES is considered the gold standard of TMB assessment but it bears noting that the size of the 'exome' depends on the enrichment method (exome capture kits) (44,45) used and that mutation types for TMB count vary between assays. Therefore, comparison of WES and panel sequencing in terms of TMB and cut-points not only requires detailed information of the panel including bioinformatics but also a clear definition of the technical reference standard (i.e., WES). For targeted gene panel sequencing in NSCLC cut-points were set at around 10 mutations per megabase (mut/Mbp) for Foundation Medicine panels (31,33) and 7.4 mut/Mpb for the MSK-IMPACT panel (29). A recent bridging study (39) using 44 samples from the Checkmate 26 trial demonstrated that the cut-point of 10 mut/Mbp (as determined by the FMI panel) relates to 199 missense mutations determined by WES. For urothelial carcinoma using the Foundation medicine (FMI) panel two different study-specific cut-points were applied with 9.65 mut/Mbp (median) (37) and 16 mut/Mbp (upper quartile) (36).

Table 1 Entity and study specific cutoffs for TMB. Overview of published studies assessing TMB

Cancer type	Study phase	Trial/drug	WES threshold (No. mut/exome)	Threshold in mut/Mbp	Percentile	RR	PFS (months)	OS (months)	Ref.
NSCLC	1/2	KN 001 pembrolizumab	200	NA	50	63% *vs.* 0%	14.5 *vs.* 3.7	NA	(6)
NSCLC	1/2	CM 012 nivolumab + ipilimumab	158	NA	50	NA	NA	NA	(28)
NSCLC	3	CM 026 nivolumab	243	NA	67	47% *vs.* 23%	HR 0.62	HR 1.10	(27)
NSCLC	1/2	MSKCC various IO therapies	NA	7.4 (IMPACT)	50	NA	38.6% *vs.* 25.1%	NA	(29)
NSCLC	1/2	CM 568 nivolumab + ipilimumab	NA	(9-)10 (F1)	50[†]	44% *vs.* 12%	7.1 *vs.* 2.6	NA	(30)
NSCLC	3	CM 227 nivolumab ± ipilimumab	NA	10 (F1)[‡]	50[†]	45.3% *vs.* 24.6%	7.1 *vs.* 3.2	NA	(31)
NSCLC	1/2	POPLAR atezolizumab	NA	9.9 (F1)	50	20% *vs.* 4%	7.3 *vs.* 2.8	16.2 *vs.* 8.3	(32,33)
SCLC	1/2	CM 032 nivolumab ± ipilimumab	248	NA	67	NA	1.3 *vs.* 1.4; 1.5 *vs.* 7.8	3.1 *vs.* 5.4; 3.4 *vs.* 22.0	(34)
BLCA	1/2	CM 275 nivolumab	167	NA	67	32% *vs.* 11%	3 *vs.* 2	11.63 *vs.* 5.72	(35)
BLCA	1/2	IMvigor210 atezolizumab	NA	16 (F1)	75	NA	NA	OS advantage	(7,36)
BLCA	3	IMVigor211 atezolizumab	NA	9.65 (F1)	50	NA	NA	HR 0.68	(37)
HNSC	1/2	KN 012 & KN 055 pembrolizumab	175	NA	NA	NA	HR 0.64	HR 0.98	(38)
SKCM	1/2	Ipilimumab/ tremelimumab	100	NA	23	NA	NA	NA	(4)

[†], according to (30); [‡], according to the bridging study (39), which employed samples of the Checkmate 26 trial, 10 mut/Mbp relate to 199 missense mutations in WES data. TMB, tumor mutational burden; BLCA, bladder urothelial carcinoma; CM, CheckMate nivolumab ± ipilimumab, Bristol-Myers Squibb; F1, FoundationOne panel; HNSC, head and neck squamous cell carcinoma; HR, hazard ratio; IMPACT, IMPACT MSKCC panel; IO Tx, immuno-oncologic therapy; KN, Keynote series of clinical trials of pembrolizumab, Merck & Co, Inc.; Mbp, megabase pairs; mut, mutations; NA, not available; NR, not reached; NSCLC, non-small cell lung cancer; OS, overall survival; PFS, progression-free survival; RR, response rate; SCLC, small cell lung cancer; SKCM, skin cutaneous melanoma.

Factors influencing TMB detection

For accurate TMB assessment pre-analytical, analytical, and post-analytical variables have to be considered and results be carefully interpreted in the overall context. Based on the current literature, five major parameters influencing TMB measurement and related cutoff values can be delineated: (I) tumor type (46,47); (II) indication including drug types; (III) pre-analytics (including assessment of tumor cellularity); (IV) method (WES *vs.* type of panel sequencing including size and composition of panels, read depth and coverage); and (V) bioinformatics (including: limit of detection (LOD), threshold for allelic frequency [clonal *vs.* subclonal mutation], and filter settings for germline events and deamination artifacts, definition of mutation). Pre-analytical factors include input material, tumor cellularity, and DNA quality and quantity. Clinical trials have mostly employed formalin-fixed, paraffin-embedded (FFPE) tissue, either fresh (obtained for study purpose) or archived (routine clinical specimens) or occasionally obtained fresh frozen tissue plus blood for germline analysis. *Table 2* gives an overview of the technical parameters of major TMB studies as provided in the respective papers. Assessment of tumor cellularity by a pathologist is warranted to ensure validity of input material (presence, percentage, and viability of tumor cells) and thereby aid subsequent interpretation of allelic frequencies. It should be noted, however, that tumor cellularity estimates itself influence allelic frequency calculations and related mutation counts (depending on LOD and filter settings). In daily clinical practice one most often has to resort to (archived) FFPE samples with usually suboptimal DNA quality (due to various degrees of DNA degradation and deamination artifacts) compared to fresh unfixed tissue. Quantities are also rather limited especially from routine clinical lung samples that are often small (transbronchial/endobronchial/image-guided) biopsies which are also used for standard pathology work-up including (limited) immunohistochemistry for tumor typing and PD-L1 staining. Most of the time this is done sequentially requiring repeated cutting of the paraffin block which causes additional tissue loss. A one-stop shop approach that yields all the required sections in one session is needed to optimize material usage (*Figure 1*). However, this will require upfront communication between clinicians, lab personnel, and pathologists, also to ensure that additional immunohistochemical and genetic studies are only performed when absolutely needed for accurate diagnosis. Looking at recent clinical trials, a rather high attrition rate of patients that could not be assessed for TMB is apparent, which might be due to sequential testing, enrichment method used (hybrid capture requires higher amount of input DNA), or unplanned addition of TMB testing. In the CM 026 trail (27), of 541 randomized patients only for 320 (59%) TMB could be assessed. Similar in the CM 227 trail (31) in which of 1,739 patients, only 1,004 (58%) had TMB-evaluable samples. Only 98 (34%) patients of 288 in the CM 568 trial had evaluable samples (30). Also in the IMvigor211 trial (37) only 544 (58%) of 931 patients had tumor samples that could be successfully tested.

In the future, blood based TMB assays might be an alternative in those clinical scenarios where tissue cannot be obtained and even more would allow for non-invasive monitoring of TMB over time. Assays are currently in development and first reports have shown correlation to TMB measured from tissue samples (48-50). However, current data are still preliminary and limited. One of the main factors influencing such approaches is the DNA yield obtained from a blood-draw: sequencing with sufficient sensitivity is well feasible but (tumor-related) DNA molecules that are not present cannot be sequenced (51).

Bioinformatics

Clinical interpretation of conventional sequencing approaches—though based on quantitative data like allele frequencies and base changes—mostly results in a qualitative result, e.g., presence or absence of a BRAF V600E mutation. In contrast, TMB as a continuous numerical value is a quantitative measurement. As such its measurement is more complex with the associated risk of capturing significant background noise. In daily clinical practice, germline samples are not routinely available for comparative sequencing due to regulatory constraints not permitting germline sequencing, logistic challenges, or financial considerations. Therefore, filtering of germline variants is paramount as not to count them as tumor specific mutations. Known polymorphisms with minor allele frequencies (MAF) <0.0001 annotated in the dbSNP and ExAC databases (52) should be filtered. Some groups have also introduced algorithms to predict germline mutations (53). Clonal hematopoiesis of indeterminate potential (Chip) might also contribute to few falsely attributed mutations in tumor-only sequencing (54) but is likely to be of only minor relevance in TMB assessment. Importantly, low allelic frequency C>T transitions that are introduced by fixation in formalin (i.e., deamination

Table 2 Technical details of TMB assessment

Cancer type	Trial/drug	Method	Input material	DNA extraction	Capture	Depth of coverage	Sequencer	Mutation types	Filtering germline	Ref.
NSCLC	KN 001 pembrolizumab	WES	FFPE or frozen	DNEasy (Qiagen)	SureSelect All Exon v2 (44Mbp) (Agilent)	164×	HiSeq 2000 (Illumina)	nonsyn.	(Blood)	(6)
NSCLC	CM 012 nivolumab + ipilimumab	WES	FFPE	NA	SureSelect All Exon v2 (44Mbp), v4 (51Mbp) (Agilent), Rapid Capture Exome target kit (38Mbp) (Illumina)	148×	HiSeq 2000, 2500, 4000 (Illumina)	nonsyn.	(Blood)	(28)
NSCLC	CM 026 nivolumab	WES	FFPE (archival)	Allprep DNA/ RNA FFPE kit (Qiagen)	SureSelect All Exon v5 (50Mbp) (Agilent)	84.6×	HiSeq 2500 (Illumina)	missen.	(Blood)	(27)
NSCLC	MSKCC various IO therapies	MSKCC	NA	DNEasy (Qiagen)	NA	744x	HiSeq 2500 (Illumina)	nonsyn.	(Blood)	(29)
NSCLC	CM 568 nivolumab + ipilimumab	FMI	NA	NA	NA	NA	NA	NA	NA	(30)
NSCLC	CM 227 nivolumab ± ipilimumab	FMI	FFPE (archival or fresh)	NA	NA	NA	NA	nonsyn. + indels	dbSNP, ExAC, FMI db, algorithm	(31)
NSCLC	POPLAR atezolizumab	FMI	NA	NA	NA	NA	NA	NA	NA	(32,33)
SCLC	CM 032 nivolumab ± ipilimumab	WES	FFPE (archival)	Allprep DNA/ RNA (Qiagen)	SureSelect All Exon v5 (50Mbp) (Agilent)	103×	HiSeq 2500 (Illumina)	nonsyn.	(Blood)	(34)
BLCA	CM 275 nivolumab	WES	FFPE (archival)	NA	NA	NA	NA	missen.	(Blood)	(35)
BLCA	IMvigor210 atezolizumab	FMI	NA	HistoGeneX NV (Antwerp)	NA	NA	NA	nonsyn. + indels + syn.	dbSNP, ExAC, FMI db, algorithm	(7,36)
BLCA	IMVigor211 atezolizumab	FMI	FFPE (archival or fresh)	HistoGeneX NV (Antwerp)	NA	NA	NA	nonsyn. + indels	NA	(37)
HNSC	KN 012 & KN 055 pembrolizumab	WES	FFPE	NA	NA	NA	NA	NA	NA	(38)
SKCM	Ipilimumab/ tremelimumab	WES	Frozen	QIAamp DNA mini (Qiagen)	SureSelect All Exon v2 (44Mbp), v4 (51Mbp) (Agilent)	>100×	HiSeq 2000 (Illumina)	nonsyn. + indels	(Blood)	(4)

TMB, tumor mutational burden; dbSNP, The Single Nucleotide Polymorphism database; ExAC, The Exome Aggregation Consortium; FFPE, formalin-fixed, paraffin-embedded tissue; FMI, Foundation Medicine panels; FMI db, Foundation Medicine proprietary internal variant database; Mbp, megabase pairs; missen., missense mutations; MSKCC, MSK-IMPACT panel; NA, not available; nonsyn., nonsynonymous substitutions; WES, whole exome sequencing.

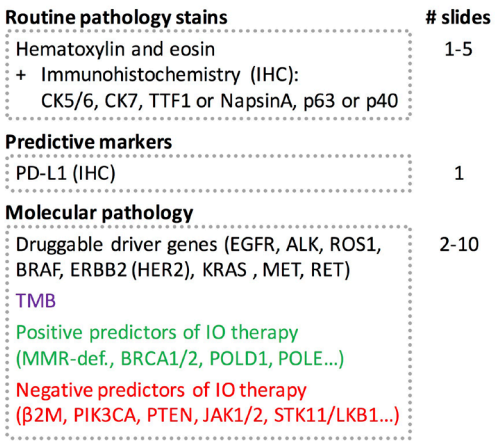

Figure 1 One-stop shop approach to maximize specimen yield. Necessary molecular testing should best be indicated by the clinician or anticipated when the specimen is initially processed in the pathology laboratory. A sufficient number of slides should be precut to avoid re-cutting of the tissue block. Depicted are three groups of diagnostic tests that are often performed sequentially. # slides: approximated number of slides needed. In routine pathology, material usage is determined by the utilization of IHC stains. In molecular pathology, the number of slides/paraffin sections needed depends on the amount of tumor present and assays used.

artifacts) need to be filtered and not mistaken as true mutational events.

FMI excludes recurrent mutations occurring in tumors and truncating mutations of tumor suppressor genes from their TMB calculation to avoid overestimation of TMB since gene panels are usually heavily targeted at recurrently mutated genomic regions (55). Our own data indicate that this is a valid approach though the influence of hot spot mutations on TMB is not prominent (Buchhalter I, 2018, submitted). Studies have used 5% allelic frequency as a cutoff to count mutations (LOD) to distinguish clonal *vs.* subclonal mutations which are entity specific and also influenced by therapy (56). In addition, patients with elevated clonal neo-antigen load and little subclonal heterogeneity were overrepresented in the group of patients benefitting from IO therapy (57). Whereas most WES studies included only non-synonymous mutations (mostly restricted to missense mutations) primarily for technical reasons (i.e., clear and reliable identification of this mutation type), panel sequencing approaches add insertions and deletions (indels) (*Table 2*). Some like FMI also include synonymous mutations, which even though they are not themselves giving rise to neoantigens, can be viewed as a surrogate marker for non-synonymous mutations elsewhere in the exome (55). Our own in-silico simulations indicate that inclusion of all mutations, even if they may not directly contribute to immunogenicity, increases precision of TMB estimation when using targeted gene panels (Buchhalter I, 2018, submitted).

Another aspect to consider is that sequencing of larger portions of the exome will result in many more detected genetic variants. How should this be dealt with in inherently resource-limited health care settings? Should all of these variants be clinically evaluated and reported? They probably will have to, which will bind additional manpower and strain IT resources. What about a potentially increased discovery rate of incidental genetic findings that might warrant genetic counselling? Refinancing of extensive manpower, reagent, and infrastructure associated costs will be challenging.

Composition and size of panel

Though one would wish to gather as much information from a patient's tumor by conducting comprehensive WES, this is currently not feasible in clinical practice due to significant associated costs, long turn-around time, and suboptimal, limited tissue samples.

Targeted panel sequencing to identify therapeutically actionable oncogenic driver genes has emerged as a routine sequencing assay in many academic centers. Given its comparatively low cost, rather quick turn-around time, and more and more widespread availability, it would be desirable to leverage it to also assess TMB. After deciding on enrichment method (amplification-based or hybrid capture) and desired read depth, the most important question arising is one of sequencing panel size: How much of the exome needs to be sequenced to make an accurate prediction of a tumor's total mutational burden (*Figure 2*)? The precision of TMB estimation is combinatorially limited by the number of bases that are sequenced. An upper limit of precision can be derived based on a model where each base in the genome is mutated at with the same probability. We calculated combinatorial confidence intervals of TMB for sequencing panels of sizes between 0.1 and 10 Mbp (*Figure 3*). Good separation of a tumor with 20 mut/Mbp from a tumor with 5 mut/Mbp was possible for panel sizes of 1 Mbp or larger.

In NSCLC and melanoma, both tumors with high mutational load mainly caused by exogenous agents tobacco and UV irradiation, respectively (58,59), cancer-type specific algorithms were reported to allow for accurate

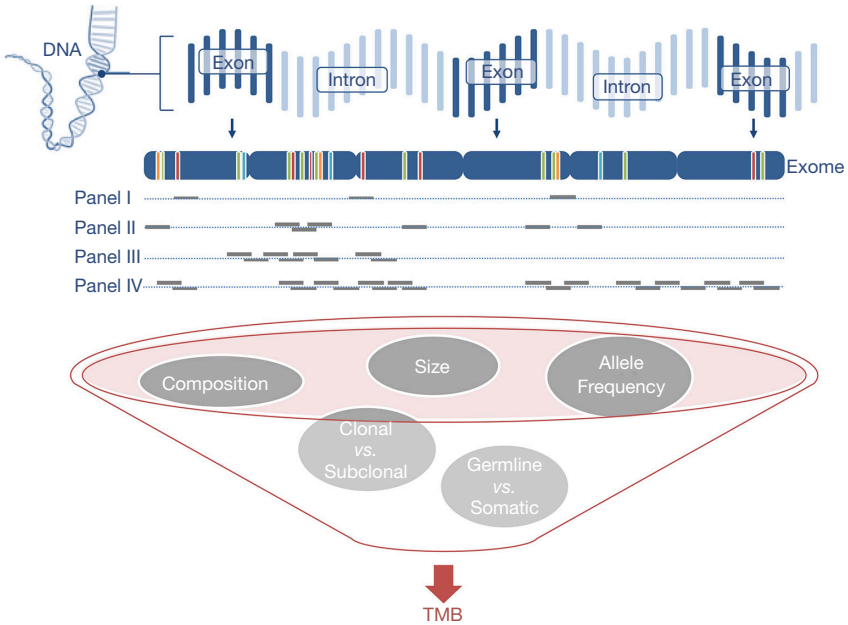

Figure 2 Panel design influences TMB measurement. Amplicons (i.e., sequenced regions of exome) of arbitrary sequencing panels (Panels I–IV) are schematically depicted to illustrate differences in size and composition. Panel I is a small focused panel which might be used for entity specific investigations or when DNA is limited, like in liquid biopsies. Panel II and III are more comprehensive targeting additional exonic regions. Panel IV is a comprehensive tumor profiling panel developed for TMB detection. Indicated on exome are exemplified clonal (green), subclonal (red), and frameshift (orange) mutations, and indels (blue). TMB, tumor mutational burden.

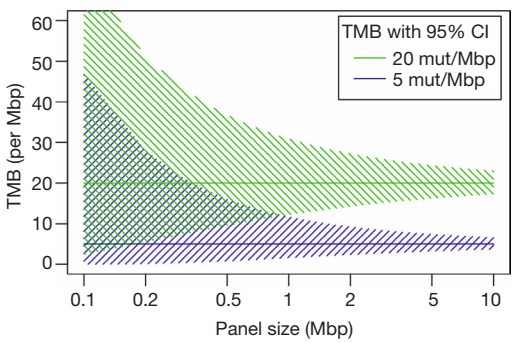

Figure 3 Precision of TMB estimation using targeted sequencing panels of size 0.1 to 10 Mbp. An upper limit for the precision of TMB estimates is set by the combinatorial error that comes from estimating the mutation rate (in mut/Mbp) by the number of mutations in a sequence of a limited length. In the display, the precision of TMB estimation (reported as 95% confidence interval) is illustrated for a tumor with high TMB (20 mut/Mbp, typically classified as immune therapy responder) and a tumor with low TMB (5 mut/Mbp, typically classified as immune therapy non-responder). Sequencing panels larger than 1 Mbp are required to separate the tumors in the example with high precision. TMB, tumor mutational burden.

prediction of TMB with a small panel of 170 genes (60). However, in clinical practice the challenge is posed by patients with intermediate TMB, accurate identification of which is difficult with small gene panels, because TMB values represent a continuum rather than distinct clusters. The 468-gene MSK-IMPACT panel (29,61) and 315-gene FoundationOne panel (62,63) have demonstrated diagnostic utility in predicting TMB comparably to WES (*Table 3*). In our experience with in-silico modeling (Buchhalter I, 2018, submitted) and resequencing of samples that previously underwent WES (Endris *et al.*, in preparation), panels covering less than 1 Mbp are not suitable for a reliable prediction of TMB, but larger panels, ideally 1.5–3 Mbp can provide a good approximation of mutational load, as shown before (68). Sequencing of larger panels will also require capable sequencing machines, like the S5XL (Thermo Fisher) or NextSeq and beyond (Illumina).

But of course, panel size is not the only factor, as composition is equally important. An ideal panel must ensure one-stop shop analysis with a combination of driver gene mutation analysis and TMB assessment to ensure maximum yield of clinically relevant information

Table 3 Side-by-side comparison of TMB assays used in currently published datasets

Features	WES	MSK-IMPACT (MSKCC)	FoundationOne CDx (FMI)
Genes	~22.000	468	324
Size	~30 Mbp[†]	1.22 Mbp	0.8 Mbp
Germline filtering	Blood	Blood	Databanks (dbSNP, ExAC, FMI internal), algorithm
TMB	Somatic, coding mutations (non-synonymous)/exome	Somatic, coding mutations (non-synonymous)/Mbp	Somatic, coding mutations (non-synonymous + indels + synonymous)/Mbp
Ref.	(64)	(6,61,65,66)	(62,67)

[†], the actual size of the 'exome' is influenced by the enrichment method (exome capture kit) used (44,45). TMB, tumor mutational burden; FMI, Foundation Medicine panels.

with limited DNA, technical resources, and economic constrains. In the case of lung cancer, this includes clinically actionable mutations in EGFR exons 19–21 including T790M alterations, as well as genetic aberrations including gene fusions that affect ALK, ROS1, RET, NTRK, BRAF, ERBB2 (HER2), KRAS, and MET (69). Furthermore, panels should ideally incorporate additional positive and negative predictors for checkpoint inhibitor therapy in order to provide a better grasp of the complex interaction between tumor and the immune system. It is of particular interest to detect tumors with defects in DNA repair genes since these tumors are prone to an increased number of somatic mutations, potentially leading to formation of tumor specific antigens, and recognition by tumor infiltrating lymphocytes. Mismatch repair-deficient tumors that are microsatellite instable (MSI-H) respond better to anti PD-1 therapy (70-72), and MSI-H status represents the first tissue-agnostic biomarker for checkpoint blockade with pembrolizumab (73,74). However, immune evasion mechanisms might still render an otherwise MSI-H tumor unresponsive to IO therapy (75,76). Defects in homologous recombination as caused by BRCA2 mutations confer a better response to checkpoint inhibitor therapy in malignant melanoma (77) and BRCA1/2-mutated high grade serous ovarian cancer exhibit a high mutational load (78). Mutations in POLD1 were noted in the lung cancer of a never-smoker with high TMB (6). POLE and POLD1 mutations occur in hypermutated colorectal and endometrial cancers (79,80). Loss-of-function mutations in chromatin remodeling gene PBRM1 sensitize tumors to IO therapy (22,81). On the flip side, loss of PTEN and subsequent activation of the PI3K-AKT pathway induced resistance to the antitumor T cell immune response in preclinical models of melanoma (82). In human lung adeno- and squamous cell carcinomas, expression of PD-L1 was found to be closely linked to mTOR activation (83). Amplification of MDM2 family proteins or aberrations in EGFR were found in patients experiencing hyperprogressive disease under treatment with checkpoint inhibitors (84) though this association was not seen in a subsequent study (29). Collectively, these data suggest that hyperprogression is still poorly understood and requires further investigation. JAK1/2 mutations as well as STAT family members were shown to confer primary resistance to PD-1 blockade (85-88). Defects in the IFN-γ pathway (89) and antigen-presenting protein beta-2-microglobulin (85,88,90,91) were shown to interfere with successful IO therapy. Patients with mutations in STK11 did not respond to treatment (28,29,92). While these data strongly suggest that the concept of negative IO-response predictors has great potential and will enter clinical stage soon, it should be noted that data of prospective clinical trials, which demonstrate the true clinical utility of these biomarkers have not been reported yet. Results from this comprehensive molecular tumor profiling should ideally be available within 10 working days from sample submission in order to ensure timely patient management.

Overall, in several retrospective and prospective clinical trials across multiple tumor entities, TMB was shown to identify patients that will or will not benefit from IO therapy more accurately than PD-L1 expression. It is therefore a promising marker that in conjunction with other parameters, like PD-(L)1 immunohistochemistry, positive and negative molecular predictors, immune cell infiltrates and inflammatory signatures can facilitate a more accurate guidance of IO interventions. However, for widespread

clinical use, harmonization of assays and reporting is crucial in order to ensure validity and comparability of results with these of clinical trials. Two major efforts are currently underway to address the issue of standardized TMB estimation:

In the United States, Friends of Cancer Research (FoCR) gathered stakeholders primarily of industry and to a lesser degree academia to conduct a three-step harmonization project (93). First, in silico TMB analysis of TCGA datasets was performed to uncover factors of variability between different assays used. Next, a reference standard employing WES will be created to enable comparison of different gene panels. Lastly, clinically meaningful cutoff values will be determined in a retrospective analysis of samples with patient outcome data.

In Germany, an academia-driven round robin test carried out by Qualitätssicherungs-Initiative Pathologie QuIP GmbH (QuIP) (94), a joint venture of the two major German pathology societies Deutsche Gesellschaft für Pathologie (DGP) and Bundesverband Deutscher Pathologen e.V. (BDP), is in progress. In contrast to the FoCR initiative this will not only involve diagnostic and pharmaceutical companies but also eleven pathology institutes across Germany and Switzerland. Testing will be carried out on human tissue samples with validation against WES data.

Since these two initiatives approach the process of harmonization from different angles, a collaboration was initiated with the intention of joining forces and laying the ground for international and cross-sector standardization of TMB measurement and reporting. Considering the importance of TMB for the complex interaction between tumors and the immune system (95), these efforts are expected to also boost our understanding of microenvironment-related biomarkers, including abundance and clonal composition of immune cell infiltrates, thus paving the way for even more individualized and effective IO therapeutic strategies.

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Footnote

Conflicts of Interest: V Endris: advisory board and lecture fees from AstraZeneca and ThermoFisher. J Leichsenring: consultancy contract with AstraZeneca. S Fröhling: speaker's honoraria from Amgen, Lilly, PharmaMar and Roche; research funding from AstraZeneca, Pfizer and PharmaMar. M Thomas: advisory board honoraria from Novartis, Lilly, BMS, MSD, Roche, Celgene, Takeda, AbbVie, Boehringer, speaker's honoraria from Lilly, MSD, Takeda, research funding from AstraZeneca, BMS, Celgene, Novartis, Roche and travel grants from BMS, MSD, Novartis, Boehringer. P Schirmacher: advisory board honoraria from Pfizer, Roche, Novartis, AstraZeneca as well as speaker's honoraria and research funding from Roche, AstraZeneca and Novartis. A Stenzinger: advisory board honoraria from BMS, AstraZeneca, Novartis, ThermoFisher, speaker's honoraria from BMS, Illumina, AstraZeneca, MSD, Novartis, Roche, ThermoFisher, and research funding from Chugai. The other authors have no conflicts of interest to declare.

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