Optimizing panel-based tumor mutational burden (TMB) measurement

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Background: Panel sequencing based estimates of tumor mutational burden (psTMB) are increasingly replacing whole exome sequencing (WES) tumor mutational burden as predictive biomarker of immune checkpoint blockade (ICB).

Design: A mathematical law describing psTMB variability was derived using a random mutation model and complemented by the contributions of non-randomly mutated real-world cancer genomes and intratumoral heterogeneity through simulations in publicly available datasets.

Results: The coefficient of variation (CV) of psTMB decreased inversely proportional with the square root of the panel size and the square root of the TMB level. In silico simulations of all major commercially available panels in the TCGA pan-cancer cohort confirmed the validity of this mathematical law and demonstrated that the CV was 35% for TMB = 10 muts/Mbp for the largest panels of size 1.1–1.4 Mbp. Accordingly, misclassification rates (gold standard: WES) to separate ‘TMBhigh’ from ‘TMBlow’ using a cut-point of 199 mutations were 10%–12% in TCGA-LUAD and 17%–19% in TCGA-LUSC. A novel three-tier psTMB classification scheme which accounts for the likelihood of misclassification is proposed. Simulations in two WES datasets of immunotherapy treated patients revealed that small gene panels were poor predictors of ICB response. Moreover, we noted substantial intratumoral variance of psTMB scores in the TRACERx 100 cohort and identified indel burden as independent marker complementing missense mutation burden.

Conclusions: A universal mathematical law describes accuracy limitations inherent to psTMB, which result in substantial misclassification rates. This scenario can be controlled by two measures: (i) a panel design that is based on the mathematical law described in this article: halving the CV requires a fourfold increase in panel size, (ii) a novel three-tier TMB classification scheme. Moreover, inclusion of indel burden can complement TMB reports. This work has substantial implications for panel design, TMB testing, clinical trials and patient management.

Key words: tumor mutational burden, TMB, immune-oncology, panel sequencing, immune checkpoint blockade

Introduction

The immune system plays a central role in cancer recognition and control. It does not only fight virus-driven tumors and limits pro-tumorigenic states of inflammation, but also monitors cells for expression of neoantigens and cell stress induced proteins [1].
patients shows good clinical response and extended progression free survival under such therapies. Due to potentially severe adverse effects [2] and substantial costs it is paramount to prospectively identify patients that most likely benefit from such treatment strategies.

Currently, PD-L1 expression as assessed by immunohistochemistry is the most widely adopted and approved predictive biomarker for ICB [3] but its predictive power, especially negative predictive value, is limited [4]. Consequently, additional markers [5–7] including tumor mutational burden (TMB) are considered. The more mutations a tumor accumulates, the higher the likelihood of production and subsequent presentation of neoantigens on major histocompatibility complex (MHC) molecules resulting in a higher likelihood of tumor cell cytotoxicity after inhibition of checkpoint signals [8]. This paradigm is supported by accumulating evidence that tumors with higher TMB are more likely to respond to ICB in various settings including PD-L1 blockade in non-small-cell lung cancer (NSCLC) [9] and urothelial carcinoma [10], CTLA-4 blockade in malignant melanoma [11, 12] and combined PD-L1-1 and CTLA-4 blockade in NSCLC [13–15] and small-cell lung cancer (SCLC) [16]. Studies have shown that TMB is to a large extent independent of PD-L1 status and might thereby identify additional subgroups of patients who benefit from ICB [13, 15, 17–19].

The definition of cut-points to separate ‘TMBhigh’ from ‘TMBlow’ tumors is not consistent in recent NSCLC trials: For example, in the CheckMate (CM) trials CM012 [15], CM227 [13] and CM026 [17] cut-points of 158 mutations, 199 mutations (estimated from a panel-based cut-point of 10 mutations per Mbp [20]) and 243 somatic missense mutations were used, respectively.

While TMB was accurately measured by whole exome sequencing (WES) in several studies, this is currently not feasible in a routine clinical setting due to high costs, long turnaround times and limited availability of sufficient tissue samples. At the same time, panel-based sequencing of routinely available formalin-fixed and paraffin-embedded tissue samples has been implemented at many clinical centers. Therefore, stakeholders of academia and industry are working on implementing assays and workflows to reliably extrapolate TMB from panel sequencing data (psTMB). Many parameters influence psTMB measurement including pre-analytical factors, the assay itself and the bioinformatics analysis pipeline [21–25].

Here, we address key issues regarding the clinical implementation of psTMB measurement: First, using a random mutation model, we derive an algebraic formula for the coefficient of variation (CV) of psTMB as a function of panel size and number of detected mutations. Secondly, by the simulation of panel sequencing in WES data, we quantify psTMB variability, assess the degree of imprecision attributable to intratumoral heterogeneity, and analyze the capability of psTMB to predict response to ICB. Thirdly, we introduce a three-tier TMB classification scheme and show how it can attenuate imprecision inherent to psTMB. Finally, we analyze how the inclusion of synonymous mutations, nonsense mutations and indels additionally to missense mutations can improve the precision of psTMB estimates.

## Materials and methods

An algebraic formula for the CV of psTMB was derived assuming binomial distribution of the number of detected mutations. Panel sequencing using five commercially available panels (Table 1) was simulated in WES data from the TCGA pan-cancer cohort, the TRACERx 100 cohort and two cohorts of ICB treated patients [13, 26–28]. Statistical computing and graphics generation was carried out with the programming language R. P-values < 0.05 were considered significant. Statistical methods are described in detail in the supplementary Appendix S1 (available at Annals of Oncology online).

## Results

### Random mutation model

Assuming that each base is mutated with the same probability, the number of mutations detected by panel sequencing is a random variable following a binomial distribution. This assumption reflects many current psTMB filtering approaches that disregard evolutionary selected genes (e.g. classic oncogenes and tumor suppressors) for TMB scores. Evaluating this binomial model, we derived a mathematical formula for the CV of psTMB: the CV inversely proportional to the square root of the panel size and inversely proportional to the square root of the TMB level. Figure 1A shows how the CV decreases when the panel size increases for tumors with TMB scores > 1, 3, 10, 30 and 100 muts/Mbp. Figure 1B shows how the CV decreases when the TMB level increases (exemplified for the major five commercially available panels investigated in the present study plus the F1 CDx panel by Foundation Medicine). For a tumor of 10 muts/Mbp, the CV of psTMB ranged between 69% and 27% for panel sizes ranging between 0.21 and 1.34 Mbp (Table 1).

### Real-world cancer genomes

Next, we simulated psTMB measurement with the five major sequencing panels in the pan-cancer TCGA cohort of > 10,000 tumors (Figure 2, supplementary Figures S1 and S2, available at Annals of Oncology online). Strong correlations were observed between the numbers of mutations detected by panel sequencing and the reference standard WES: Pearson correlation reached 0.95 for the OCAv3 panel, 0.97 for the TST170 panel and 0.99 for the three large panels (Figure 2A, D and G). Linear models were fitted to analyze the correlation of panel-approximated TMB and WES-measured TMB. As expected, the values for the slopes reflected the ratio of the panel size to the size of the sequence region covered by WES (supplementary Figure S2, available at Annals of Oncology online). Additionally, we detected intercepts ranging from 0.82 to 1.23 mutations, which were substantially different from the unbiased situation of an intercept of zero. This means that typically about one additional mutation was detected by panel-based sequencing in addition to what would be expected by multiplicative scaling, a bias that reflects the enrichment of typical panel designs for frequently mutated cancer genes. The variance of the residuals in the linear fit of psTMB versus WES-TMB showed a linear increase with the number of mutations.
Table 1. Precision of TMB measurement of five commercially available sequencing panels

<table>
<thead>
<tr>
<th>Panel acronym</th>
<th>Panel name</th>
<th>Provider</th>
<th>Panel size (Mbp)</th>
<th>CDS covered by panel (Mbp)</th>
<th>CV (random mutation model) for TMB = 10 muts/Mbp (%)</th>
<th>Additional CV contribution from real-world cancer exomes (%)</th>
<th>CV (real-world cancer exomes) for TMB = 10 muts/Mbp (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSO500</td>
<td>TruSight oncology 500</td>
<td>Illumina</td>
<td>1.95</td>
<td>1.34</td>
<td>27</td>
<td>28</td>
<td>35</td>
</tr>
<tr>
<td>QIAseq</td>
<td>Human tumor mutational burden panel</td>
<td>Qiagen</td>
<td>2.58</td>
<td>1.26</td>
<td>28</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>OTML</td>
<td>Oncomine tumor mutational load assay</td>
<td>Thermo Fisher Scientific</td>
<td>1.66</td>
<td>1.18</td>
<td>29</td>
<td>17</td>
<td>34</td>
</tr>
<tr>
<td>TST170</td>
<td>TruSight tumor 170</td>
<td>Illumina</td>
<td>0.53</td>
<td>0.41</td>
<td>49</td>
<td>21</td>
<td>60</td>
</tr>
<tr>
<td>OCAv3</td>
<td>Oncomine comprehensive assay v3</td>
<td>Thermo Fisher Scientific</td>
<td>0.35</td>
<td>0.21</td>
<td>69</td>
<td>28</td>
<td>88</td>
</tr>
</tbody>
</table>

The CV (coefficient of variation) of the panel TMB (tumor mutational burden) score was calculated in a random mutation model and estimated in simulations in the real-world cancer exomes (TCGA pan-cancer data). The numbers of mutations in the CDS (coding sequence) regions covered by the panels were compared with the total number of mutations in the CDS.
These data demonstrate that the mathematical law derived in the random mutation model—a decrease of the CV of the panel TMB estimate with the square root of the TMB level—remained valid for real-world cancer exomes. Of note, the CV in the real-world situation was 17%–28% higher than in the random mutation model (Figure 2C, F and I; Table 1).

To analyze the influence of selected genes on the variance of psTMB we simulated three different kinds of panels: (i) panels exclusively composed of oncogenes and tumor suppressor genes, (ii) panels of randomly drawn genes, (iii) panels of randomly drawn genes excluding oncogenes and tumor suppressor genes. Again, the mathematical law before could be confirmed (supplementary Figure S3, available at Annals of Oncology online).

The additional variance adding to the one predicted by the random mutation model was approximately twice as high (15%) for panels composed of oncogenes and tumor suppressor genes compared with the other panels (6%).
Intra-tumor inhomogeneity of psTMB

We compared the TMB in different regions of 100 tumors of the TRACERx lung cancer cohort [27]. TMB and simulated psTMB were calculated including the missense mutations with variant allele frequency (VAF) greater or equal to 5% (Figure 3A). Analyzing a TMB cut-point of 199 mutations, 11% of the 100 tumors were classified inconsistently by different regions. Classification with psTMB resulted in higher inconsistency rates of 18%, 16%, 17%, 15% and 24% for the TSO500, QIAseq, OTML, TST170 and OCAv3 panels, respectively; however, these differences reached significance only for one of the smaller panels: OCAv3 (P = 0.026). Next, we quantified the intra-tumor variation of TMB by calculating the CV between the regions of each of the 100 tumors (Figure 3B–D, supplementary Figure S4, available at Annals of Oncology online). The average CV of the cohort was substantially higher for all panel estimations of TMB (23.1%, 24.1%, 24%, 27.6% and 28.2% for TSO500, QIAseq, OTML, TST170 and OCAv3, respectively) compared with the WES calculation of TMB (15.3%).

Prediction of response to IO therapy

We analyzed two clinically annotated cohorts of ICB treated patients with tumors characterized by WES [13, 28].

In the Miao et al. dataset [28], we compared the capability of psTMB to separate responders (CR/PR, n = 70) from patients with progressive disease (PD, n = 123). The analysis was carried out in the subcohort of lung cancer patients (n = 36), melanoma patients (n = 125) as well as in the mixed cohort types (five cancer types, n = 193) for which WES data were available (Figure 4).

In lung cancer, high TMB was strongly predictive of response to ICB: areas under the curve (AUC) were between 0.78 and 0.94. Tumor classification by psTMB and tumor indel ratio

In addition to missense mutations, we analyzed synonymous mutations, nonsense mutations and indels and calculated the ratio of the number of the latter mutations to the number of the former mutations for each of the tumors in the TCGA pan-cancer cohort (Figure 6). Indel burden in conjunction with either high or low TMB identified specific tumor types and genetic subgroups including MSI-H tumors which are known to respond well to ICB (for details see supplementary Appendix SR.2 and Figure S6, available at Annals of Oncology online).

Three-tier versus two-tier TMB classification

In the TCGA data, we analyzed the feasibility to classify tumors by panel sequencing compared with the gold standard of WES. The four ‘WES thresholds’ 158 muts, 199 muts (equivalent to 10 muts/Mbp [20]), 243 muts and ‘median TMB’ were converted to corresponding ‘panel thresholds’ using a linear transformation (supplementary Table S1, available at Annals of Oncology online). The median TMB was analyzed additionally to the clinical validated thresholds, because for many cancer types very few tumors had TMB above these thresholds (supplementary Table S2, available at Annals of Oncology online). For most of the cancer types, misclassification rates were substantially lower when using the three larger panels (with panel sizes > 1 Mbp) compared with the two smaller panels (Figure 5A and supplementary Table S2, available at Annals of Oncology online); however, even with the three large panels, misclassification rates (exampled for a threshold of 199 mutations) were substantial: 17%–19% for lung squamous cell carcinoma (LUSC), 10%–12% for lung adenocarcinoma (LUAD), 7%–11% for cutaneous melanoma (SKCM) and 5%–6% in the pan-cancer cohort. To deal with the substantial fraction of misclassified tumors, we studied a refined classification approach of replacing the cut-point by a three-tier classifier that is based on the likelihood of misclassifications (Figure 5B). The interval width was determined in such a way, that the percentage of strong misclassifications (tumor classified as ‘TMBhigh’ by panel sequencing, when harboring low TMB and vice versa) was <5%. The interval width was substantially smaller for the three larger panels compared with the two smaller panels and smaller in the pan-cancer cohort compared with the lung cancer and melanoma subcohorts (Figure 5C–F).

Discussion

The predictive power of TMB as biomarker for response to ICB is currently investigated in many clinical trials [22, 23] across various cancer types. At first WES was widely used to determine TMB, but now there are a growing number of clinical studies which interrogate subsets of the genome by gene panels to approximate TMB (psTMB) [22, 23]. While in the clinical trial context these analyses are mainly carried out by commercial providers, many clinical laboratories depending on the regulatory
**Figure 3.** Inhomogeneity of tumor mutational burden (TMB) across different regions of lung tumors TRACERx 100 data [27]. (A) TMB of 323 regions of 100 lung carcinoma measured by WES and classification using a cut-point of 199 mutations. Regions of the same tumor were classified inconsistently for 11 tumors (red dots). (B–D) The coefficient of variation (CV) of TMB across regions was calculated for each of the tumors. The CV was substantially higher in simulated panel sequencing data (TSO500, QIAseq and OTML panels) compared with the WES data.
Approval context will eventually use pre- or self-designed gene panels to determine TMB scores.

TMB is a continuous variable and cut-points that discriminate between likely ICB responders and likely non-responders have to be investigated in clinical trials separately for each cancer type; however, many parameters influence TMB measurement and thus the TMB score for an individual tumor. In this work, we describe key influencing factors that are inherent to the nature of the analytical design of psTMB testing and need to be carefully considered when implementing any TMB assay in the clinic. Our analysis lays the foundation for future studies that will investigate additional parameters influencing wet-lab performance (e.g. pre-analytical factors, enrichment and sequencing technologies [25]), which is beyond the scope of this work. Several consortial approaches are under way to address these issues [29]; however, while careful control of wet-lab parameters may minimize their influence and potential bias, the methodological limitations described in this article will remain unaffected by any of these efforts and apply to any panel.

Analyzing a stochastic mutation model, we observed that the variability of TMB counts (CV) is an algebraic function of panel size and TMB. Specifically, the CV is inversely proportional to the square root of the panel size and inversely proportional to the square root of the TMB of the tumor. Practically speaking, a scenario with low cut-points and TMB scores determined by small gene panels will result in a high imprecision of TMB measurement and will not reliably identify patients who benefit from ICB. For a tumor with a TMB close to the cut-point of 10 muts/Mb the CV turned out to be 22%, 26%, 32%, 45% and 63% for sequencing with panel sizes of 4, 2, 1, 0.5 and 0.25 Mbp. Translating this
data to a concrete real-world case, let us assume a trial enrollment scenario where a patient’s tumor has a true TMB score of 15 and 10 muts/Mb is used as the cut-point separating TMBhigh from TMBlow patients. When TMB is estimated by a 2 Mbp panel the CV value mentioned above corresponds to a CI of 10.1–21.4 muts/Mbp meaning that there is a strong likelihood that this patient will be enrolled into the TMBhigh group. Now let us consider two slightly different scenarios where a 1 or a 0.5 Mbp panel was used to estimate TMB for the very same patient: the corresponding CIs would be 8.4–24.7 muts/Mbp and 6.3–30.2 muts/Mbp, respectively. In the latter scenarios one cannot exclude that this patient although belonging to the TMBhigh group would be...

Figure 5. Analysis of the cut-point of 199 missense mutations to separate ‘TMBhigh’ from ‘TMBlow’ tumors. Simulations in the TCGA lung squamous cell carcinoma (LUSC), lung adenocarcinoma (LUAD), cutaneous melanoma (SKCM) and pan-cancer cohorts. (A) Numbers of misclassified tumors (in %) using tumor mutational burden (TMB) measurements of five commercial sequencing panels. (B) Introduction of a three-tier scheme to keep the rate of strongly misclassified tumors (classified as TMBhigh instead of as TMBlow and vice versa) under control. (C–F) The interval width was determined in such a way that strong misclassifications (classification above the gray by panel sequencing, while the tumor is classified below the gray area by WES and vice versa) occurred for <5% of the tumors.
Figure 6. Analysis of other mutation types in relation to missense mutations. (A, C, E) Scatterplots showing strong correlations of synonymous mutations and nonsense mutations with missense mutations, but moderate correlation of indels with missense mutations. (B, D, F) Violin plots showing the ratio of numbers of a specific mutation type to missense mutations.
enrolled into the TMBlow group based on test results. This can evidently influence trial results but can also affect individual patient management outside clinical trials. In summary, the inherent imprecision of psTMB estimates drastically increases for panel sizes <1 Mbp. Simulation of panels in the TCGA dataset showed that this algebraic law remained valid in real-world cancer genomes with CVs (and corresponding CIs) that are even larger than in the random mutation model.

For only a few cancer types, including colorectal cancer, stomach cancer and uterine corpus endometrial carcinoma, TMB shows a bi- or multimodal distribution [30]. In these cancers, the TMB distribution is shaped by the occurrence of hypermutation in MMR deficient and/or POLE/POLD1 mutated tumors and permits a clean dichotomization; however, for most of the other cancer types including lung adenocarcinoma, lung squamous cell carcinoma and cutaneous melanoma, TMB is unimodally distributed with a dense point cloud of TMB scores scattering around the cut-point. For accurate classification of these tumors, TMB scores need to be determined by gene panels of a considerable size to obtain reliable results, i.e. with an acceptable CV. While we acknowledge that the CV will never be zero, it is evident that uncontrolled high CV values derived from insufficiently powered gene panels can misclassify individual patients. Moreover, it can lead to false assignment to TMB groups in clinical trials and thus impact clinical trial datasets as well as subsequent treatment guidelines.

Employing two datasets [13, 28], we showed that the AUC of psTMB substantially differs from WES-TMB particularly when using small gene panels [31] leading to substantial clinical misclassifications. This scenario can be controlled by establishing a three-tier classification that depends on the likelihood of misclassification instead of a single cut-point. Setting the error margin for cases which were designated TMBhigh whereas they were TMBlow in reality (and vice versa) at 5%, a subset of patients is identified where the panel itself operates with such substantial variability that clinically meaningful assignment of an individual patient to either one of the groups (low/high) is not possible—even though a cut-point has demonstrated clinical utility when analyzing a cohort of patients in a clinical trial. Introducing this concept of a ‘gray zone’ of TMB values would provide a safety margin in which clinicians could weigh in additional factors to their decision making (i.e. co-morbidities, other treatment options). This concept is transparent for clinicians as it conveys which TMB values to take for certain and which might be more error prone, a concept proposed for quantitative diagnostic tests before [32].

psTMB scores are also influenced by regional sampling of the tumor further contributing to the imprecision of psTMB when considering an individual case detailed above. This is a substantial finding as psTMB will be determined in biopsy material from stage IV lung cancer patients reflecting a proportion but not the entirety of the tumor, and is in line with seminal work on intratumoral genetic heterogeneity [33–35].

Both synonymous mutation burden and nonsense mutation burden turned out to be proportional to tumor (missense) mutation burden for the majority of tumors; however, in line with seminal work by Turajlic et al. [36], our work highlights indel burden as an independent parameter in the mutational spectrum of TMB. We found that the proportion of indels (in relation to missense mutations) in conjunction with either high or low TMB values identifies different tumor types and genetic subgroups including MSI-H cases which are well known to respond to ICB [37].

In conclusion, our work shows that a universal mathematical law describes an imprecision inherent to psTMB, which is independent of pre-analytics and specific analysis parameters (e.g. coverage) and can influence patient management and clinical trial results. This scenario is further aggravated by spatial inhomogeneity of psTMB scores. Panel designs that consider the mathematical law described in this article as well as a novel three-tier classification system can control for the variable precision of psTMB. Moreover, our analysis suggests that indel burden can complement TMB results. These findings have implications for panel designs, psTMB testing and clinical decision making.

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