Original Research

Assessments of tumor mutational burden estimation by targeted panel sequencing: A comprehensive simulation analysis

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Impact statement

This study addresses a crucial aspect in the field of cancer immunotherapy by quantitatively evaluating the variances of TMB values calculated by whole exome sequencing (WES) and targeted panels. As TMB emerges as an indicative factor for immune checkpoint inhibitor sensitivity and targeted panel sequencing has been increasingly used, the accurate estimation of TMB becomes critical. By conducting a comprehensive investigation using 10,000 simulated targeted panels with varying sizes, we demonstrate the impact of panel size on TMB estimation. In addition, we identify high-impact gene sets for different cancer types, highlighting the cancer type–specific effects on TMB estimation. These findings provide valuable insights into the potential use of targeted panels for TMB assessment, presenting a cost-effective and efficient alternative to WES. Our work contributes to advancing personalized treatment decisions in cancer patients based on directly measured TMB from sequencing data obtained with targeted panels.

Abstract

Tumor mutational burden (TMB), when at a high level, is an emerging indicative factor of sensitivity to immune checkpoint inhibitors. Previous studies have shown that the more affordable and accurate targeted panels can be used to measure TMB as a substitute for whole exome sequencing (WES). However, additional processes, such as hotspot mutations exclusion and TMB adjustment, are usually required to deal with the effect of the limited panel sizes. A comprehensive investigation of the effective factors is needed for accurate TMB estimation by targeted panels. In this study, we quantitatively evaluated the variances of TMB values calculated by WES and targeted panels using 10,000 simulated targeted panels with panel sizes ranging from 0.2 to 3.1 million bases. With The Cancer Genome Atlas (TCGA) cancer samples and mutation profiles, we fixed regressions on WES-TMBs and panel-TMBs to assess the performance of a given targeted panel. Panel size was found as one of the major effective factors of TMB estimation. Meanwhile, by investigating the well-performing small panels that reported TMB values similar to those of WES, we demonstrated the evidence of the cancer type–specific impacts of genes on TMB estimation and identified high-impact gene sets for different cancer types based on the TCGA data. This study revealed the quantitative correlations between TMB variance and panel size, and the potential impacts of individual genes on TMB estimation. Our results suggested that for cancer patients diagnosed using targeted panels, it would be highly beneficial to have the capability to directly measure TMB from the targeted sequencing data. This would greatly assist in making decisions regarding the use of immunotherapies.

Keywords: Tumor mutational burden, TMB, targeted panel sequencing, simulation analysis

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Introduction

Immune checkpoint inhibitors have demonstrated improved survival for multiple cancers.[1–3] However, patients' responses are highly variable. Currently, programmed death ligand 1 (PD-L1) expression and microsatellite instability are approved by the Food and Drug Administration (FDA) as biomarkers for immunotherapy.[4] It is not a perfect biomarker that some patients tested positive for PD-L1 but not respond to the therapy, and some negative tested patients may still respond.[5–7] Hence, other predictable indicators are needed. Tumor mutational burden (TMB) is a promising indicator that can discriminate responders across several tumor types.[8–10] Gandara *et al.*[11] used the blood-based assay to measure TMB in plasma and found the high TMB to be clinically actionable.

TMB is calculated as the total number of somatic mutations per mega-base in coding regions for a tumor sample. TMB has been found to have a remarkable association with tumor immune response and has gained more interest in cancer studies. Usually, tumors with high-TMB levels are more likely to respond to immunotherapy.[12] In many clinical

studies, WES is considered the gold standard for estimating TMB in research. Recently, deep-targeted panel sequencing, which can detect mutations with higher accuracy and is cost-effective and less time-consuming, has drawn a lot of attention.[13] Studies have shown high correlation in TMB measured by WES and targeted panels.[14,15] Xu *et al.*[16] built a pan-cancer for TMB estimation by selecting some genes with high mutation frequencies from different cancer types. Their optimized panel indicated high correlation with WES.

TMB estimation using targeted panels has been found to exhibit biases against WES, leading to various efforts to reduce the differences between these two approaches. An association between WES- and panel-TMB variance and panel size has been reported. In short, compared to WES, bigger panels performed better in measuring TMB. Chalmers *et al.*[14] found that below 0.5 Mbps, the variance in TMB measurement increased significantly and claimed that targeted panels of 1.1 Mbps would be sufficient for measuring TMB. Researchers also have focused on other potential factors that may affect TMB estimation, such as the top mutated genes and hotspot mutations.[16] Heydt *et al.*[15] measured the correlation of five large panels with WES for TMB estimation and found that including or excluding synonymous variants had little influence on correlation. By measuring a large number of tumor samples, the authors also identified a novel mutation hotspot in the *PMS2* gene that may be associated with increased TMB in skin cancer.[14] However, the current outcomes were not significant enough to guide the design and application of targeted panels for TMB.

In this study, we quantitatively investigated the effect of panel size on TMB estimation by simulating 10,000 targeted panels with a large range of sizes (0.2–3.1 Mbps). Different metrics confirmed that, on average, panels larger than 1 Mbps could output TMB compatible with that of WES. We then investigated the outliers (badly performing large panels and well-performing small panels) and observed the impacts of individual genes on TMB estimation. A big panel can show very high-TMB variance if many high-impact genes are covered. In addition, we found the gene impact on TMB was cancer type specific.

Materials and methods

TMB calculation

Within the TCGA MAF file, a total of 2,899,874 PASS mutations from 9018 samples in CDS regions were used for the TMB estimation in this study. A TMB value was calculated as the number of mutations per million base pairs covered by the interested genome regions.

$$TMB = \frac{Number\ of\ Mutations}{Region\ Size} \times 1{,}000{,}000$$

The WES-TMB was evaluated for all CDS regions, which was more than 32 Mbps, and all the mutations in CDS regions were included in the calculation. On the contrary, the region size of a given targeted panel was the part of the panel in CDS regions. Only the mutations covered by the CDS part of the panel were taken.

Panel simulation processes based on COSMIC genes

All COSMIC genes were obtained from the webpage (*https://cancer.sanger.ac.uk/census*). The genes in the Cancer Gene Census tier1 and tier2, which are strongly indicative of some roles in cancer were separated from the others. After the exclusion of genes outside the CDS regions, 557 tier1, 139 tier2, and 9021 other genes remained for the targeted panel simulation. With the average gene size around 2.9 Kbps, we simulated 10,000 panels by selecting from 70 to 1100 genes to cover a large range of panel sizes. To mimic the designed targeted panels that focused more on cancer-associated genes, we used the gene selection strategy to build the panels:

1. When $N \leqslant 400$ ($N \geqslant 70$), N is the total number of genes selected, the ratio of tier1 and tier2 genes was 3:1, and no other gene was selected.
2. When $N > 400$ ($N \leqslant 1100$), we always selected 100 tier2 genes. The rest of the genes were from tier1 and others with the ratio 1:1.
3. All genes were randomly selected from the given pool.

For example: When N = 100, #T2 = 25, #T1 = 75, #Other = 0. When N = 600, #T2 = 100, #T1 = 250, #Other = 250. When N = 1,100, #T2 = 100, #T1 = 500, #Other = 500.

Only the CDS parts of the genes were used to simulate panels. Finally, we generated a bed file for each of the panels and bedtools[17] was used to sort and merge the genome regions of the selected genes. As a result, panels from 0.2 Mbps to 3.1 Mbps were generated (Figure 2(b)).

Calculation of RMSD, FNR, and MCC

The RMSD was used to evaluate the differences between WES-TMBs and panel-TMBs for a group of TCGA cancer samples:

$$RMSD\left(P_j\right) = sqrt\left[\frac{\sum_{i=1}^{n}\left(TMB_{WES.i} - P_j\left(TMB_{Panel.i}\right)\right)^2}{N}\right]$$

where P represents the panels and i is an individual sample for which the TMB is calculated. The P_j calculates the panel-TMB of sample i. N represents the number of samples in the group for assessment.

The FNR was calculated under the detective TMB threshold as 10, showing that this patient should benefit from the immunotherapy. For a given sample with the WES-TMB greater than 10, a false-negative (FN) detection was found if the panel reported the TMB as less than 10. In this study, the total sample number was 1945 (samples with WES-TMB between 5 and 30), used as the denominator when calculating the rate.

To measure the MCC, WES-TMB 10 was used as the cutoff to classify positive ($TMB > 10$) and negative ($TMB \leqslant 10$) samples. Each sample was then classified based on the panel-TMBs. Next, the true-positive (TP), true-negative (TN),

a

Panel	Size in CDS
AGL	2,635,700
UNI6	1,344,477
BRP	1,043,782
MSK	896,895
QGN	764,916
ILM	406,619
IDT	409,996
IGT	299,005

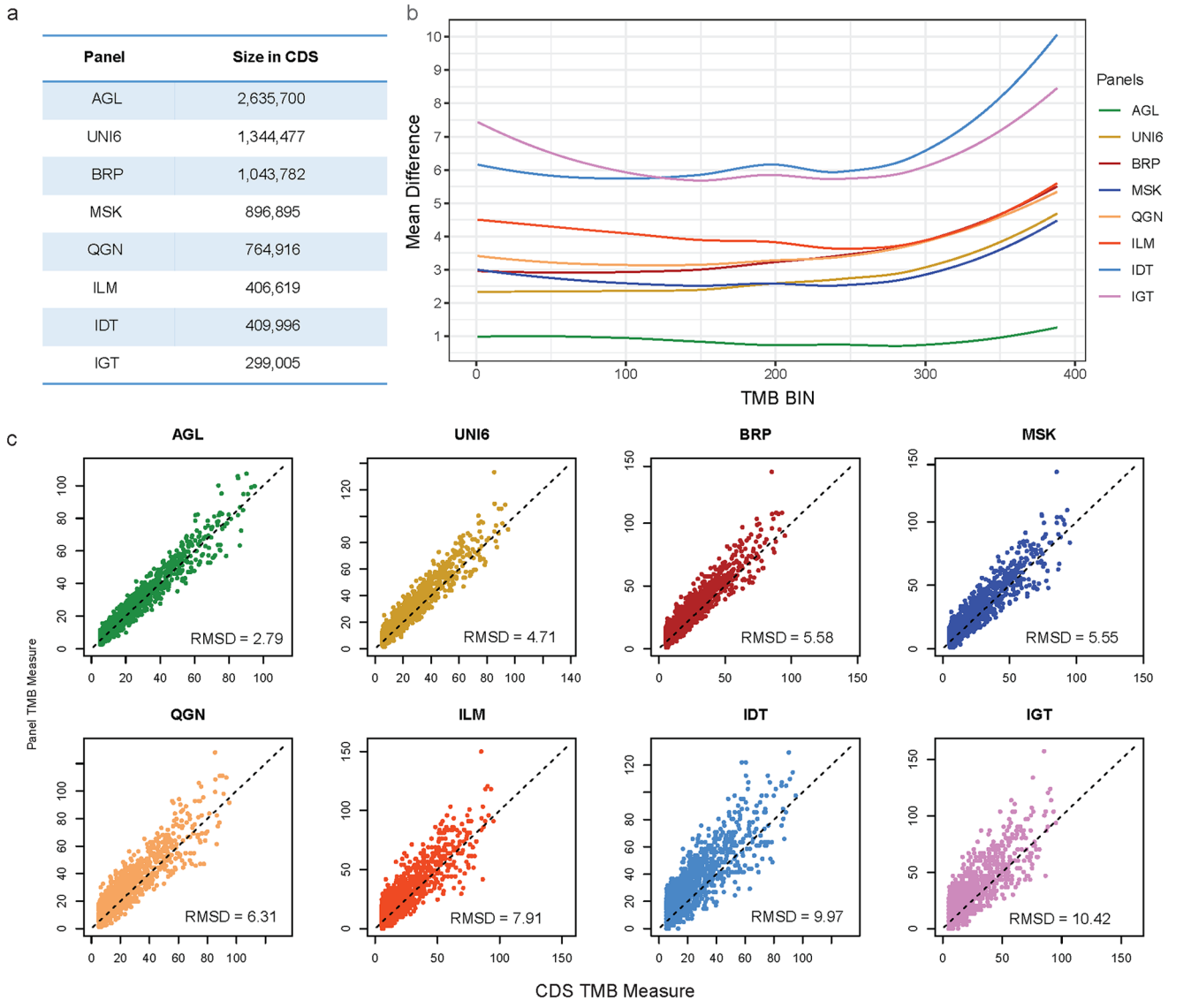

Figure 1. Targeted panel-TMB evaluation using TCGA mutation annotation. (A) Targeted panels with various sizes involved in our study. (B) Mean difference between TMBs measured on panel and CDS regions. Samples were sorted by TMB and 10 samples with closest TMBs were grouped together to calculate a mean TMB value. We then took five samples per step to generate the TMB bins to display. Two adjacent bins shared 5 samples. (C) Scatter plot of TMB measures of individual samples. RMSD was calculated using the samples with CDS-based TMB levels between 5 and 30. Panel codes: AGL: Agilent ClearSeq Comprehensive Cancer Panel v2, BRP: Burning Rock DX OncoScreen Plus, MSK: Memorial Sloan Kettering MSK-IMPACT 13, QGN: Qiagen Comprehensive cancer panel, ILM: Illumina TruSight Tumor 170, IDT: Integrated DNA Technologies xGen Pan-Cancer Panel, IGT: iGeneTech AIOnco-seq.

false-positive (FP), and FN rates were calculated to further measure the MCC, as below:

$$MCC = \frac{TP \times TN - FP \times FN}{\sqrt{(TP+FP)(TP+FN)(TN+FP)(TN+FN)}}$$

Individual gene impact on TMB estimation

To assess the potential impact of a given gene *G* on TMB estimation, we calculated the new TMB after *G* was removed from the gene list and compared it with the original TMB based on the TCGA cancer samples. To calculate the new TMB without *G*, the mutations called in *G* were removed from the total mutations of a cancer sample and the size of this gene was also taken from the total CDS size. For a given sample S, the potential impacts of *G* were estimated as:

$$TMB.diff = \left| TMB_{WES} - TMB_{G-removed} \right|$$

$$TMB.diff.ratio = \frac{\left| TMB_{WES} - TMB_{G-removed} \right|}{TMB_{WES}}$$

$$TMB_{G-removed} = \frac{\left| Total\ \#\ Mutations - \#\ Mutations\ in\ G \right|}{Size_{CDS} - Size_{G}}$$

Results

Targeted panels, in comparison to WES, reported greater TMB values by panel size

To investigate the patterns and effective factors of TMB estimation by targeted panels in comparison to WES, we first downloaded the mutation profile from The Cancer Genome Atlas (TCGA) in Mutation Annotation Format (MAF, https://

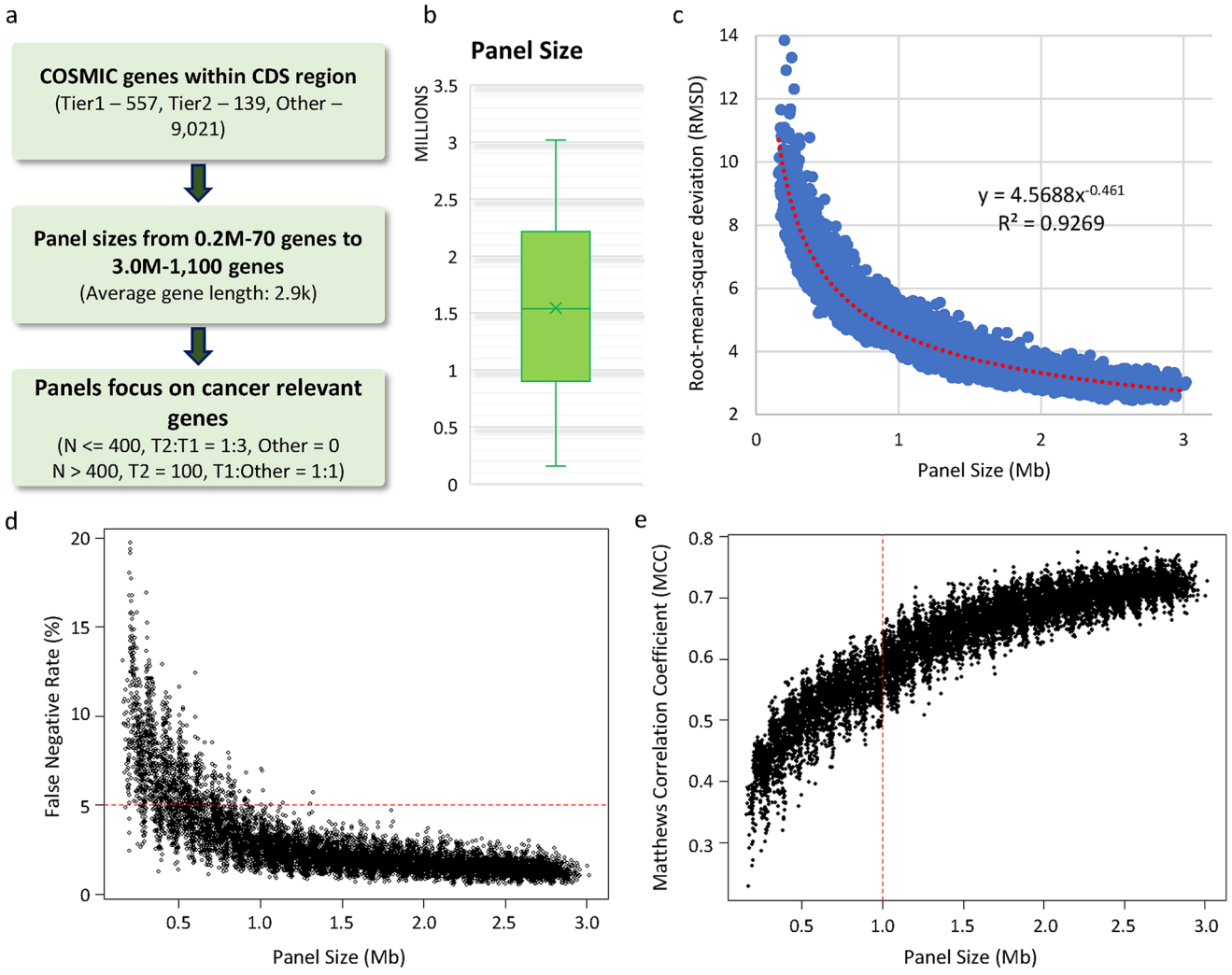

Figure 2. Assessment of 10,000 simulated panels using COSMIC genes. (a) Simulation workflow. (b) Panel size distribution. (c) Root-mean-square deviation (RMSD) measured between TMB by panels and CDS. (d) False-negative rate for identification of responsible patients. (e) Matthews Correlation Coefficient (MCC) measurement given TMB 10 as threshold, positive if TMB $>$ 10, negative when TMB $\leqslant$ 10.

gdc.cancer.gov/about-data/publications/mc3-2017). Only the PASS mutations in coding sequence (CDS) regions were used to estimate the WES-TMBs. For a given TCGA cancer sample, the WES-TMB value was calculated by dividing the number of a sample's mutations by the size of the entire CDS region in million base pairs (Methods). Considering that a medium level (about 10^{16}) of TMB is being used as the biomarker for immunotherapy, 1945 TCGA cancer samples with WES-TMB values between 5 and 30 were included in this study. These samples were enriched with the cancer types LUSC, LUAD, SKCM, and BLCA (Supp. Fig. 1). We then collected seven widely used targeted panels (Figure 1) and took the CDS regions only to estimate the panel-TMBs. The panel sizes, in CDS regions, were from 0.3 to 2.6 million base pairs (Figure 1(A)). We also created a medium-sized panel by merging the six smaller panels, named UNI6, together, which was 1.34 Mbps (Figure 1(A)). We then calculated the panel-TMB values of the 1945 TCGA cancer samples and compared them with the WES-TMBs (Methods).

We found that, on average, smaller panels tended to report higher TMBs, but the difference between panel-TMB and WES-TMB were roughly consistent across different TMB levels (Figure 1(B) and Supp. Fig. 2). Other studies also reported the pattern of higher TMB values from small panels,[14] suggesting the potential mutation enrichment of some cancer-relevant genes frequently covered by targeted panels.

Figure 1(C) shows the TMB estimation shifting between targeted panels and WES. Larger root mean square deviations (RMSDs) were found with decreasing panel sizes (Methods). Results indicated that panel size is one of the major effective factors when applying targeted panels for TMB evaluation; large differences are expected with small panels. On the contrary, with very similar panel sizes, IDT, 409,996 bps, and ILM, 406,619 bps, the mean RMSDs were very different (IDT 9.97 vs ILM 7.91). The results showed that the genes the targeted panels covered may also have significant impact on TMB estimation.

Comprehensive investigation of TMB estimation via simulation analysis

To better understand the effects of panel size as well as the genes targeted by panels on TMB estimation, we simulated 10,000 targeted panels consisting of a large range of numbers

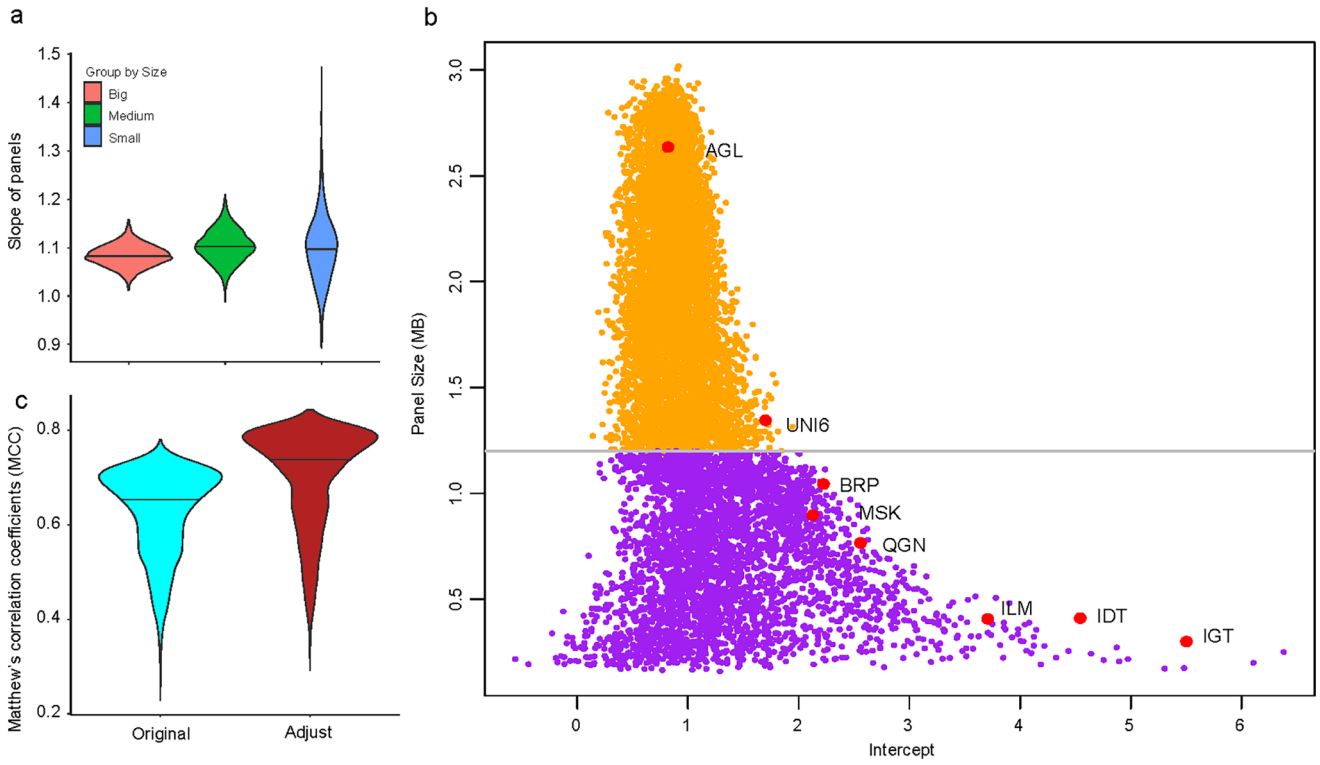

Figure 3. Assessment of the correlation between TMB evaluation and panel size by regression analysis. (a) Distribution of the slopes of three groups of various-sized panels. Small group, panel size < 1M; medium group, 1M ⩽ panel size < 2M; big group, panel size > 2M. (b) The scatter plot of intercepts fitted by regression and panel sizes. Intercepts of the eight targeted panels were added (red). (c) Comparison of the MCCs calculated with original and adjusted TMBs.

of Catalog Of Somatic Mutations In Cancer (COSMIC) genes.[18] To mimic the real-world design of targeted panels, tier1 and tier2 COSMIC genes which are more relevant to cancers were selected with higher probabilities compared to those of the other COSMIC genes during the simulation processes (Figure 2(A), Methods). As a result, 10,000 panels that targeted from 70 genes (panel size 0.2 Mbps) up to 1100 genes (3.0 Mbps) were generated (Figure 2(B)).

For each simulated panel, the panel-TMB values of the 1945 TCGA cancer samples with the WES-TMBs between 5 and 30 were calculated. Next, we compared these panel-TMBs and WES-TMBs by the RMSD, false-negative rate (FNR), and Matthew's correlation coefficients (MCC) (Methods). Along with the increased panel sizes, we obtained smaller RMSDs, lower FNRs (if a sample with WES-TMB was higher than the threshold but the panel-TMB was lower), and higher MCCs (which measure the performance of classifying the samples into low- or high-TMB groups using targeted panels) indicating the better performance by larger panels (Figure 2(C) to (E), Methods). More specifically, patients with high-TMB levels, (e.g. 10), should be identified correctly because they are most likely to benefit from the immune therapy. A lower FNR represents less chance that we missed this patient for the treatment. Taken together, panels of approximately one million base pairs and larger are suitable for TMB evaluation and would result in reliable concordance with those of WES.

We observed a pattern of TMB value shift, on average, between panel- and WES-TMBs, which was highly correlated with panel size from Figure 1(C). To closely investigate this TMB shift, we then applied linear regressions to investigate the relationships between panel- and WES-TMBs. For each panel, 1945 panel-TMBs were used to fit a linear regression model to describe the TMB correlations.

$$TMB_{Panel.i} = \alpha_i \times TMB_{WES} + \beta_i (i = 1, 2, 3, \ldots, 10{,}000)$$

With the 10,000 simulated panels, 10,000 slopes (α_i) and intercepts (β_i) were calculated. Consistent with our observations using the eight targeted panels (Figure 1(C)), the slopes were tightly clustered around 1.1, especially for bigger panels (Figure 3(A)). Next, the intercepts versus panel sizes were plotted for representing the panel-based TMB estimation shift from WES. The shifts were convergent around 1 with the increasing panel sizes (>1.2 M, orange dots). The shifts of smaller panels (⩽1.2 M, purple dots) were more spread out (Figure 3(B)). The eight panels' intercepts were embedded into the plot (red dots). We found that, with the exception of AGL, the other targeted panels had large intercepts compared to most of the simulated panels of similar sizes.

Notably, many small panels (<1.2 Mbps) also resulted in reasonable small TMB shifts. We then separated the small panels into little-shift ($0.5 < \beta \leqslant 1.5$) and massive-shift ($\beta > 1.5$) groups to discover the differences. Further investigation of the genes that made up the panels showed that TP53, which usually contains a large number of mutations, were highly presented (74.1%, 984/1328) in the massive-shift group compared with the little-shift panels (6.8%, 149/2196, Supp. Fig. 3). More than 35,000 TP53 mutations were identified by previous studies in various cancer types and cell lines,[19] and most cancer samples present some amount of

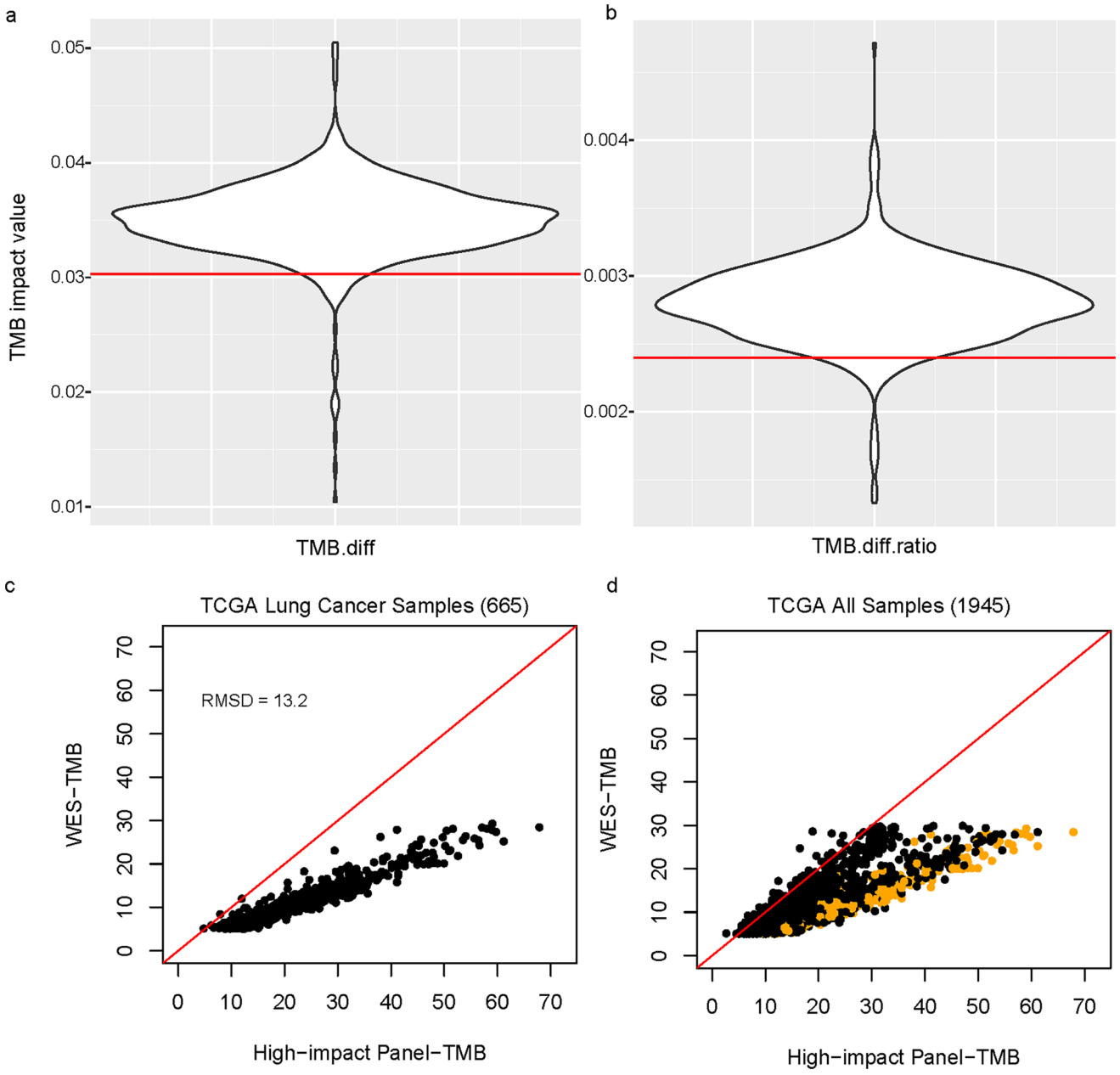

Figure 4. The average gene impacts on TMB estimation. For the genes that impacted at least 45 samples, the majority of them had the impact values greater than the median ((A) TMB.diff. (B) TMB.diff.ratio). (C) TMB comparison between WES and the "high-impact panel" on the 665 TCGA LUSC and LUAD samples (RMSD = 13.2). Even though this panel is so big, over 4 Mbps, we still observed a large difference in TMB estimation. (D) TMB comparison between WES and the "high-impact panel" on the 1945 TCGA samples. TCGA LUSC and LUAD samples were colored as orange.

TP53 mutations. We found that the majority of the TCGA cancer samples in this study contained one to three mutations within the CDS region of TP53, resulting in a TMB shift compared to WES-TMBs.

Therefore, given the purpose of a targeted panel for TMB evaluation in clinical applications, we could fix a regression model using the large number of TCGA cancer samples and mutations to estimate the performance of the targeted panel and measure the variation between panel-TMB and WES-TMB. The same method could also be used to compare multiple targeted panels for TMB estimation. Basically, compared with WES-TMB estimation, panels commonly have a consistent scale of 1.1 and a shift of around 1 (big panels). Large shifts are expected for small panels (Figure 3(B)). By adjusting the panel-TMB as below, we can obtain a closer assessment of WES-TMB measurement. For example, the median MCC was increased to 0.741 from 0.655 with the adjusted panel-TMB (Figure 3(C), Supp. Fig. 4).

$$Adj.TMB_{Panel} = \frac{TMB_{Panel} - 1}{1.1}$$

Genes targeted by panels differently impact TMB estimate

Our previous results showed that same-size targeted panels may report TMBs with large deviations from each other,

potentially due to the different genes covered by the panels. To assess the impacts of individual genes on TMB estimation, we calculated the TMB difference by original WES and a particular gene excluded from the gene list. Genes are different by cancer types in terms of mutation type and density. We therefore first took the TCGA LUSC and LUAD samples with original WES-TMB between 5 and 30 (655 samples) for the analysis.

We calculated the TMB.diff and TMB.diff.ratio of more than 9000 genes that contain at least 10 mutations across all TCGA lung cancer samples. The TMB.diff was the absolute TMB value difference before and after a given gene was excluded. TMB.diff.ratio was calculated as TMB.diff divided by original TMB-WES (Methods). As a result, taking all non-zero impact values of all samples into account, we observed median TMB.diff as 0.0303 and median TMB.diff.ratio as 0.0024. We then calculated the average gene impact on TMB over the 665 TCGA lung cancer samples. Only the non-zero values were used in the calculation. We kept and sorted the genes that impacted at least 45 samples (non-zero values in at least 45 samples) and obtained 746 high-impact genes in total. The top 30 genes and the impact values are listed in Table1. Many genes, such as *TTN, CSMD3, LRP1B, RYR2, ZFHX4*, and *USH2A* impacted TMB estimation with both TMB.diff and TMB.diff.ratio. The violin plots (Figure 4(A) and (B)) showed that most of these genes' average TMB impact was greater than the median values.

To further confirm that these genes could heavily impact TMB estimation, we built a "high-impact panel" that targeted the 746 genes (4.18 Mbps in CDS) and calculated the TMBs using this panel. We applied the high-impact panel to the TCGA lung cancer samples and to all samples (WES-TMB between 5 and 30), respectively. As shown in Figure 4(C), the panel-TMB values based on this panel were much higher than those of the WES-TMBs. The RMSD was 13.2 calculated from the TCGA lung cancer samples. Even though this panel was extra large, the RMSD was just similar to targeted panels between about 0.25 Mbps (Figure 2(C)), suggesting these genes had high impacts on TMB estimation. Notably, in Figure 4(D), TCGA lung cancer samples were clearly split from other samples based on the TMB differences by the high-impact panel. The results also suggested the gene impact on TMB was cancer type specific.

In addition, we simulated samples with fixed TMB levels based on high-TMB TCGA samples and an artificial reference sample with a high density of mutations. The mutations included in these simulated samples were randomly selected. Interestingly, the calculated TMBs of these samples were lower than the TMB in the artificial reference sample (Supp. Fig. 5 and Discussion). The results further indicated that individual genes could have varying impacts on TMB estimation. As for a cancer genome, the possibilities of the mutations in different genes were not the same; therefore, a random selection would not reflect the true distribution of mutations.

Table 1. Top 30 impact genes.

Gene name	TMB.diff	Gene name	TMB.diff.ratio
TTN	0.050484	TTN	0.004715
CSMD3	0.049889	CSMD3	0.003955
LRP1B	0.0491	LRP1B	0.003933
RYR2	0.048078	RYR2	0.003847
ZFHX4	0.047281	NFE2L2	0.003845
USH2A	0.043838	ZFHX4	0.003792
KCNC2	0.042625	FAM135B	0.003738
FAM135B	0.042417	STK11	0.003694
SPTA1	0.042396	SPTA1	0.003624
CDH10	0.042327	OR2M2	0.003538
CCT8L2	0.041785	CDH10	0.00353
FLG	0.041688	MUC16	0.003511
PAPPA2	0.041418	USH2A	0.003494
TRIM58	0.041215	SLITRK2	0.003456
SLIT3	0.040907	PTEN	0.003401
ZNF804A	0.040905	OR2M7	0.003377
MUC16	0.04084	CNTN3	0.003373
CNTN3	0.040788	OR4C12	0.003334
PCDH15	0.040677	ATM	0.003332
FAM47C	0.040674	ZNF804A	0.003324
CNTNAP4	0.040641	NALCN	0.003319
SPEF2	0.040509	OR2L8	0.003317
OR8I2	0.040428	KRAS	0.003294
KCNA4	0.040367	PRDM9	0.00329
SLITRK2	0.040351	TP53	0.003287
MYH15	0.040296	PIK3CA	0.003287
ZNF536	0.040231	THSD7A	0.003287
PCDH11X	0.040218	ZNF521	0.003278
NALCN	0.04018	C6orf118	0.003274
ASTN1	0.040177	CSMD1	0.003254

TMB: tumor mutational burden.

Discussion

In this study, we used 10,000 simulated targeted panels with a large range of sizes to quantitatively investigate the potential contributing factors of TMB estimation. We confirmed that larger panels would perform well with small TMB variations in many cases. However, many outliers, well-performing small panels and big panels with large variations based on TCGA mutation data, were also observed. We showed that the choice of the targeted genes by panels was even more critical than panel size. A big panel may show large variations if many high-TMB-impact genes were selected. Furthermore, we showed that high-TMB-impact genes were cancer type specific. A targeted panel designed for other purposes, such as driver mutation detection, may not be suitable for TMB estimation. With a given targeted panel, use of a number of WES cancer samples, (e.g. TCGA), to assess performance before putting it to clinical use is recommended.

We showed that *TP53* was targeted by many low-performing small panels (<1.2 Mbps), significantly higher than in well-performing small panels. We further investigated the medium-size panels (1.2–2 Mbps), which are generally considered well-performing panels and found that 44% of them covered *TP53*. With increased panel sizes, the effect of individual genes could be diluted by the big denominator in the TMB calculation. That is also why compatible panel-TMBs were observed by big panels. However, if a targeted panel incidentally consisted of many high-TMB-impact genes, the panel size would not help with the TMB estimation.

To investigate the sample-to-sample variance when measuring TMB by targeted panels, we then performed further analyses by simulating cancer samples with a fixed TMB level, such as 15. First, we collected 89 TCGA cancer samples with high WES-TMBs that are greater than 100 (Supp. Fig. 6). To perform the simulation, we randomly selected 482 mutations from each of these high-TMB TCGA samples that made the simulated TMB values of each sample equal to 15. Multiple samples were generated based on one high-TMB TCGA sample and the numbers of samples we simulated were determined by the total number of mutations in the TCGA samples. We simply generated more samples from a TCGA sample with more mutations:

$$Number\ of\ Samples = round(\frac{N}{482} \times 3)$$

where N is the total number of mutations in the given sample. For instance, the smallest N of these samples that could generate 6.8 (3272/482) samples without overlapped mutations was 3272. We then simulated three times more samples by allowing overlap of mutations. Therefore, this TCGA sample was used to simulate 20 new samples with fixed TMB equal to 15. As a result, 4643 samples with WES-TMB as 15 were simulated for the analysis. The eight targeted oncopanels (Supp. Fig. 5a) were then used to calculate the panel-TMBs of these 4,643 samples. Bigger panels resulted in tighter ranges of panel-TMBs around the median value than did smaller panels. Interestingly, the median TMBs were consistent (11–12, Supp. Fig. 5a) across all the panels, but smaller than the values in theory (15).

We next simulated cancer samples based on reference DNA sequencing data generated in our previous studies.[20,21] The variants detected by the six targeted panels AGL, BRP, QGN, ILM, IDT, and IGT directly[21] were used for TMB calculation. With the extra high variant density in the reference sample, which means a high level of TMB, we randomly selected 1% from the whole pool of known variants predetected each time and simulated 10,000 samples with fixed TMB level equal to 19. We then calculated the TMBs of each simulated sample by the six targeted panels and found the same patterns; output of panel-TMBs with a median of 15, lower than the theoretical TMBs, which were less than 19 (Supp. Fig. 5b). Also, we noticed that the random selection flattened the various impacts of mutated genes. In other words, mutations in a real tumor sample do not present with uniform possibilities.

AUTHORS' CONTRIBUTIONS

DL and JX proposed the original idea for the study. DL conducted the analyses and prepared figures. DW, DJ, and HH provided input and ideas for the analyses. All authors participated in writing and revising the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: This article reflects the views of its authors and does not necessarily reflect those of the U.S. Food and Drug Administration. Any mention of commercial products is for clarification only and is not intended as approval, endorsement, or recommendation.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Dobry AS, Zogg CK, Hodi FS, Smith TR, Ott PA, Iorgulescu JB. Management of metastatic melanoma: improved survival in a national cohort following the approvals of checkpoint blockade immunotherapies and targeted therapies. *Cancer Immunol Immunother* 2018;**67**:1833–44
2. Bodor JN, Boumber Y, Borghaei H. Biomarkers for immune checkpoint inhibition in non–small cell lung cancer (NSCLC). *Cancer* 2020;**126**:260–70
3. Ghatalia P, Zibelman M, Geynisman DM, Plimack ER. Checkpoint inhibitors for the treatment of renal cell carcinoma. *Curr Treat Options Oncol* 2017;**18**:1–14
4. Fashoyin-Aje L, Donoghue M, Chen H, He K, Veeraraghavan J, Goldberg KB, Keegan P, McKee AE, Pazdur R. FDA approval summary: pembrolizumab for recurrent locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma expressing PD-L1. *Oncologist* 2019;**24**:103–9
5. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, Hassel JC, Rutkowski P, McNeil C, Kalinka-Warzocha E. Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med* 2015;**372**:320–30
6. Gandini S, Massi D, Mandalà M. PD-L1 expression in cancer patients receiving anti PD-1/PD-L1 antibodies: a systematic review and meta-analysis. *Crit Rev Oncol Hematol* 2016;**100**:88–98
7. Yi M, Jiao D, Xu H, Liu Q, Zhao W, Han X, Wu K. Biomarkers for predicting efficacy of PD-1/PD-L1 inhibitors. *Mol Cancer* 2018;**17**:1–14
8. Marabelle A, Fakih M, Lopez J, Shah M, Shapira-Frommer R, Nakagawa K, Chung HC, Kindler HL, Lopez-Martin JA, Miller WH, Jr, Italiano A, Kao S, Piha-Paul SA, Delord JP, McWilliams RR, Fabrizio DA, Aurora-Garg D, Xu L, Jin F, Norwood K, Bang YJ. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. *Lancet Oncol* 2020;**21**:1353–65
9. Chan TA, Yarchoan M, Jaffee E, Swanton C, Quezada SA, Stenzinger A, Peters S. Development of tumor mutation burden as an immunotherapy biomarker: utility for the oncology clinic. *Ann Oncol* 2019;**30**:44–56
10. Qiu P, Poehlein CH, Marton MJ, Laterza OF, Levitan D. Measuring tumor mutational burden (TMB) in plasma from mCRPC patients using two commercial NGS assays. *Sci Rep* 2019;**9**:114

11. Gandara DR, Paul SM, Kowanetz M, Schleifman E, Zou W, Li Y, Rittmeyer A, Fehrenbacher L, Otto G, Malboeuf C, Lieber DS, Lipson D, Silterra J, Amler L, Riehl T, Cummings CA, Hegde PS, Sandler A, Ballinger M, Fabrizio D, Mok T, Shames DS. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. *Nat Med* 2018;**24**:1441–8
12. Goodman AM, Kato S, Bazhenova L, Patel SP, Frampton GM, Miller V, Stephens PJ, Daniels GA, Kurzrock R. Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancerstmb predicts response to immunotherapy in diverse cancers. *Mol Cancer Ther* 2017;**16**:2598–608
13. Merino DM, McShane LM, Fabrizio D, Funari V, Chen SJ, White JR, Wenz P, Baden J, Barrett JC, Chaudhary R, Chen L, Chen WS, Cheng JH, Cyanam D, Dickey JS, Gupta V, Hellmann M, Helman E, Li Y, Maas J, Papin A, Patidar R, Quinn KJ, Rizvi N, Tae H, Ward C, Xie M, Zehir A, Zhao C, Dietel M, Stenzinger A, Stewart M, Allen J TMB Harmonization Consortium. Establishing guidelines to harmonize tumor mutational burden (TMB): in silico assessment of variation in TMB quantification across diagnostic platforms: phase I of the Friends of Cancer Research TMB Harmonization Project. *J Immunother Cancer* 2020;**8**:e000147
14. Chalmers ZR, Connelly CF, Fabrizio D, Gay L, Ali SM, Ennis R, Schrock A, Campbell B, Shlien A, Chmielecki J, Huang F, He Y, Sun J, Tabori U, Kennedy M, Lieber DS, Roels S, White J, Otto GA, Ross JS, Garraway L, Miller VA, Stephens PJ, Frampton GM. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med* 2017;**9**:34
15. Heydt C, Rehker J, Pappesch R, Buhl T, Ball M, Siebolts U, Haak A, Lohneis P, Buttner R, Hillmer AM, Merkelbach-Bruse S. Analysis of tumor mutational burden: correlation of five large gene panels with whole exome sequencing. *Sci Rep* 2020;**10**:11387
16. Xu Z, Dai J, Wang D, Lu H, Dai H, Ye H, Gu J, Chen S, Huang B. Assessment of tumor mutation burden calculation from gene panel sequencing data. *Onco Targets Ther* 2019;**12**:3401–9
17. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 2010;**26**:841–2
18. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, Boutselakis H, Cole CG, Creatore C, Dawson E. COSMIC: the catalogue of somatic mutations in cancer. *Nucleic Acids Res* 2019;**47**:D941–7
19. Edlund K, Larsson O, Ameur A, Bunikis I, Gyllensten U, Leroy B, Sundström M, Micke P, Botling J, Soussi T. Data-driven unbiased curation of the TP53 tumor suppressor gene mutation database and validation by ultradeep sequencing of human tumors. *Proc Natl Acad Sci* 2012;**109**:9551–6
20. Jones W, Gong B, Novoradovskaya N, Li D, Kusko R, Richmond TA, Johann DJ, Bisgin H, Sahraeian SME, Bushel PR. A verified genomic reference sample for assessing performance of cancer panels detecting small variants of low allele frequency. *Genome Biol* 2021;**22**:1–38
21. Gong B, Li D, Kusko R, Novoradovskaya N, Zhang Y, Wang S, Pabón-Peña C, Zhang Z, Lai K, Cai W. Cross-oncopanel study reveals high sensitivity and accuracy with overall analytical performance depending on genomic regions. *Genome Biol* 2021;**22**:1–23

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