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ORIGINAL ARTICLE

Aligning tumor mutational burden (TMB) quantification across diagnostic platforms: phase II of the Friends of Cancer Research TMB Harmonization Project

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Background: Tumor mutational burden (TMB) measurements aid in identifying patients who are likely to benefit from immunotherapy; however, there is empirical variability across panel assays and factors contributing to this variability have not been comprehensively investigated. Identifying sources of variability can help facilitate comparability across different panel assays, which may aid in broader adoption of panel assays and development of clinical applications.

Materials and methods: Twenty-nine tumor samples and 10 human-derived cell lines were processed and distributed to 16 laboratories; each used their own bioinformatics pipelines to calculate TMB and compare to whole exome results. Additionally, theoretical positive percent agreement (PPA) and negative percent agreement (NPA) of TMB were estimated. The impact of filtering pathogenic and germline variants on TMB estimates was assessed. Calibration curves specific to each panel assay were developed to facilitate translation of panel TMB values to whole exome sequencing (WES) TMB values.

Results: Panel sizes >667 Kb are necessary to maintain adequate PPA and NPA for calling TMB high versus TMB low across the range of cut-offs used in practice. Failure to filter out pathogenic variants when estimating panel TMB resulted in overestimating TMB relative to WES for all assays. Filtering out potential germline variants at >0% population minor allele frequency resulted in the strongest correlation to WES TMB. Application of a calibration approach derived from The Cancer Genome Atlas data, tailored to each panel assay, reduced the spread of panel TMB values around the WES TMB as reflected in lower root mean squared error (RMSE) for 26/29 (90%) of the clinical samples.

Conclusions: Estimation of TMB varies across different panels, with panel size, gene content, and bioinformatics pipelines contributing to empirical variability. Statistical calibration can achieve more consistent results across panels and allows for comparison of TMB values across various panel assays. To promote reproducibility and comparability across assays, a software tool was developed and made publicly available.

Key words: precision medicine, biomarker, tumor mutational burden, immunotherapy, cancer

INTRODUCTION

The use of anti-programmed death-ligand 1 (PD-L1)/anti-programmed cell death protein 1 (PD-1) therapies has risen dramatically over the last few years, with an increasing number of regulatory approvals in several cancer types.[1]

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Despite these successes, only a fraction of cancer patients benefit from immune checkpoint blockade, which has led to broader exploration of biomarkers to identify patient populations more likely to respond to immunotherapy.[2-4]

Tumor mutational burden (TMB) is defined as the number of somatic mutations per megabase of interrogated genomic sequence. There has been early success in using TMB to predict responses to immune checkpoint inhibitors for patients with melanoma and lung cancer, among others.[5-7] Importantly, the use of TMB as a biomarker is tumor agnostic. Recently, data from KEYNOTE-158 (NCT02628067) supported the use of pembrolizumab for the treatment of TMB-high adult and pediatric patients with unresectable or metastatic solid tumors that had progressed after previous treatment. TMB high was set at TMB $\geq$10 mut/Mb for patients' formalin-fixed paraffin-embedded (FFPE) tumor tissue samples tested with the Foundation Medicine (Cambridge, MA) FoundationOne CDx assay.[8] The findings of this study led to the first United States Food and Drug Administration (FDA) approval of pembrolizumab using TMB high as a positive predictive biomarker for patient selection in a tissue-agnostic setting. The FoundationOne CDx assay is the first FDA-approved companion diagnostic to measure TMB and to help identify patients who may be appropriate for treatment with pembrolizumab, regardless of solid tumor type.

In clinical practice, next generation sequencing (NGS) targeted gene panel assays are preferred over whole exome sequencing (WES) approaches for TMB estimation due to already relevant clinical use for identification of targetable oncogenes, broader availability, quicker turnaround time, and cost. To date, FoundationOne CDx is the only FDA-approved panel assay that reports TMB, while the Memorial Sloan Kettering Cancer Center MSK-IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets), NantHealth's Omics Core test, and the PGDx elio™ tissue complete assay have received FDA 510(k) clearance.[9] However, access to these regulated panel assays is not ubiquitous, leading to the development of additional NGS targeted gene panel assays by local and commercial providers.

Several factors impact variation among panel assays including sample input, tumor content, panel size, gene content, quality control (QC), NGS platform, and bioinformatics pipeline, which may influence TMB estimates and lead to inconsistent TMB calculation and reporting.[10] Although 10 mut/Mb is the cut-off for TMB-high designation with the FoundationOne CDx assay, other panels may have different clinical cut-offs. Because of these inherent differences, the standardization of clinical validation practices, harmonization of TMB assessment, and alignment across TMB panel assays are critical steps to improve consistency of results and comparability across panel assays, and to promote confidence in the use of this biomarker. This is a crucial time to seek harmonization in TMB measurement and assess comparability across TMB assays to prevent the inconsistencies seen in past biomarkers. For example, the lack of alignment across PD-L1 immunohistochemistry assays, lack of comparability of panel assay results, and different cut-offs defined for each drug have posed a significant challenge for the implementation of PD-L1 expression testing.[11-17]

With the aim of facilitating harmonization and alignment across tissue TMB assays, the Friends of Cancer Research (Friends) TMB Harmonization Consortium was formed. The TMB Consortium, which consists of several diagnostic manufacturers, academics, pharmaceutical companies, the National Cancer Institute (NCI), Frederick National Laboratory for Cancer Research, and the FDA, previously reported results from the first phase of the project where the theoretical variability across 11 commercial and academic panel assays was described and consortium-endorsed recommendations were proposed for the analytical validation of TMB assays.[18] Moreover, the TMB Consortium partnered with Quality in Pathology (QuIP) in Germany to complement its approach and enrich its perspective on the variability in TMB estimates across laboratories through a technical comparability study.[19]

In this study, we set out to characterize the empirical variability in TMB measurements across platforms using a common set of cell lines and clinical samples tested across 16 panel assays from 16 participating laboratories. Further, we aimed to elucidate how certain factors such as panel size, gene content, and bioinformatics pipelines impact TMB estimates, and to investigate the use of a calibration tool based on The Cancer Genome Atlas (TCGA) data and human tumor-derived cell lines that will facilitate comparability across different panel assays. Based on these results, we aim to provide data and guidance that will help improve the consistency and reliability of panel tissue TMB estimation across platforms and facilitate the use of this complex biomarker in clinical decision making.

MATERIALS AND METHODS

Samples (clinical samples and cell lines)

Thirty-six FFPE clinical tumor samples and matched buffy coat were acquired from iSpecimen (Lexington, MA) and processed at a reference laboratory (MoCha Laboratory, Frederick National Laboratory for Cancer Research, Frederick, MD), where tumor tissue specimens were enriched by histological macrodissection to the extent possible, with the estimated tumor cell content in macrodissected specimens ranging from 30% to 95% (Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2021.09.016). All samples were categorized into the following broad tumor types: bladder, colon, gastric, gastrointestinal stromal tumor (GIST), and lung. Specific histologic diagnoses and demographic data can be found in Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2021.09.016. Genomic DNA from tumor specimens was extracted using AllPrep FFPE Nucleic acid Extraction kit and the QIAcube automated platform (QIAGEN, Germantown, MD). Genomic DNA from buffy coat specimens was extracted using the QIAsymphony automated platform (QIAGEN). DNA was quantitated using Qubit dsDNA BR assay kit (Thermo Fisher

Scientific, Waltham, MA). After performing QC, seven GIST samples were excluded from further analyses mostly due to low DNA yield, poor DNA quality, and low depth of coverage (Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2021.09.016); thus, only 29 clinical samples were evaluated in this study.

Ten (two breast, eight lung cancer) human-derived matched tumor-normal cell lines were selected and obtained from the American Type Culture Collection (ATCC) (Supplementary Table S3, available at https://doi.org/10.1016/j.annonc.2021.09.016) and processed at a reference laboratory, SeraCare (now LGC Clinical Diagnostics Division). Cell lines were grown in accordance with ATCC specifications with no more than five passages. DNA was extracted from frozen cell pellets (80-100M cells) using the QIAGEN Gentra Puregene Kit. Purified genomic DNA concentrations were normalized to 50 ng/μL in 0.1x Tris-EDTA buffer as measured by the Qubit dsDNA BR assay kit. Integrity of purified genomic DNA was assessed by agarose gel electrophoresis. All 10 matched cell line samples passed QC, and thus were evaluated in this study.

Whole exome sequencing and TMB estimation

The reference laboratory carried out WES, where 50 ng of genomic DNA was sheared to 150-180 bp using Covaris LE220 sonicator (Covaris, Woburn, MA). Library preparation was automated on a SciClone G3 liquid handling workstation using custom scripts (Supplementary Material, available at https://doi.org/10.1016/j.annonc.2021.09.016). A NovaSeq 6000 (Illumina, San Diego, CA) was used with 2 × 150 bp paired-end (PE) sequencing mode. WES TMB was calculated using the previously described uniform method using two Novaseq S4 flowcells generating ∼400M PE 150-bp reads on tumor and ∼135M reads on normal samples to generate a median target coverage of >400× in tumor and >200× in normal tissue.[20] GATK-based Sentieon pipeline (version v201808) was used to call somatic variants (https://github.com/FNL-MoCha/nextgenseq_pipeline).

Gene panel assay sequencing and TMB estimation

Aliquoted DNA samples extracted from clinical samples and cell lines were distributed to all 16 participating laboratories, and each used their own sequencing and bioinformatics pipelines to estimate TMB from the genes represented in their respective panel assays. Some of these pipelines have been previously published (Table 1). Clinical samples were run as singletons and cell lines were run in duplicate or triplicate as available.

Panel assay size analysis

The simulated positive percent agreement (PPA) and negative percent agreement (NPA) of each of the panel assays (*in silico*) were calculated as a function of both the size of the panel assay used for its calculation as well as the respective TMB cut-off (Supplementary Material, available at https://doi.org/10.1016/j.annonc.2021.09.016).

Panel gene content analysis

Ten laboratories volunteered their BED file formats to anonymously evaluate the gene content of their panel assays. All panel data were lifted over to hg19 coordinates if they were not already. The intervals in these panel assays were intersected with the xgen-exome-research-panel-v2-targets exome reference panel assay. TCGA mutations from WES (in MAF format) were then overlayed on to the panel assays. We explored the removal of variants flagged as pathogenic as per the Catalogue of Somatic Mutations in Cancer (COSMIC) version 88, as well as synonymous variants, to determine the impact of including or excluding certain variants. TMB estimates per sample and per gene were tabulated.

Germline analysis

Three laboratories that use a tumor-only approach for the removal of germline variants volunteered to estimate the TMB value of the 29 clinical samples using three specific population minor allele frequency (pMAF) thresholds (0%, 0.5%, and 1%) to assess the impact that different population pMAF thresholds have on TMB estimates. Each laboratory used their own combination of population allele databases, including some custom databases, but no additional methods for the removal of germline variants were used (i.e. custom copy number-based germline prediction methods). (Supplementary Material, available at https://doi.org/10.1016/j.annonc.2021.09.016).

Calibration analysis

Statistical analyses were conducted to develop calibration curves specific to each panel assay that would facilitate translation of panel TMB values to WES TMB values. For each panel assay, two potential calibration curves were constructed. One curve modeled the association between panel TMB and WES TMB based on *in silico* analysis of the TCGA validation data as previously described.[18] These WES TMB values, which were previously calculated, are available on Precision FDA (https://precision.fda.gov/). The second curve modeled the association based on the "wet lab" results obtained on 10 human tumor cell lines newly generated and reported on in the current article. Parameter estimates were then used to compute 95% prediction limits. WES-calibrated TMB estimates and 95% intervals of uncertainty were obtained by inverting the fitted regression line and prediction limits. Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2021.09.016, provides a pictorial representation of the calibration process. Additional documentation describing details of the model fit, calculation of the prediction limits, and method of obtaining the WES-calibrated TMB estimates and intervals of uncertainty can be found in Supplementary Material, available at https://doi.org/10.1016/j.annonc.2021.09.016.

After fitting the calibration curves for each panel assay according to the TCGA and cell line methods (training sets), the calibration curves were applied to TMB measurements generated by the panel assay on a completely independent

Table 1. Characteristics of 16 participating diagnostic NGS panel assays

Factor type	ACT	AZ	BWH	Caris	FMI	ILLUM	IPG	JHU	MSKCC	NeoGenomics	OmniSeq	PGDx	Q2	QIAGEN	Thermo_OCA	Thermo_OTMLA
Panel assay characteristics																
Name of panel assay	ACTOnco+	AZ650	OncoPanel v3.1	SureSelectXT	F1 CDx	TSO500	TheraMap Solid Tumor (TSO500)	JHOP2	MSK-IMPACT	NeoTYPE Discovery Profile for Solid Tumors	Ion AmpliSeq Comprehensive Cancer Panel	PGDx elio tissue complete	TSO500	QIAseq TMB panel	Oncomine Comprehensive Assay Plus (OCA Plus)	Oncomine Tumor Mutation Load Assay (OTMLA)
Number of genes	440	649	447	592	324	523	523	432	468	372	409	505	523	486	517	409
TMB region covered	1.1 Mb	1.65 Mb	1.94 Mb	1.40 Mb	0.8 Mb	1.33 Mb	1.27 Mb	1.14 Mb	1.14 Mb	0.935 Mb	1.17 Mb	1.3 Mb	1.2 Mb	1.33 Mb	1.06 Mb	1.2 Mb
Processing																
Minimum DNA input	40 ng	100 ng	50 ng	50 ng	50 ng	40 ng	40 ng	50 ng	150 ng	20 ng	30 ng	50 ng	40 ng	40 ng	20 ng	20 ng
Quantification method	Fluorescence	Fluorescence	Fluorescence	Electrophoresis	Fluorescence	Fluorescence	Fluorescence	Electrophoresis	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence
Technology uses UMIs	No	Yes	No	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes	Yes	No	No
Deduplication	No	Yes, UMI based	Yes, not UMI based	Yes, not UMI based	Yes, UMI based	Yes, UMI based	Yes, UMI based	Yes, not UMI based	Yes, not UMI based	Yes, UMI based	No	Yes, UMI based	Yes, UMI based	Yes, UMI based	No	No
Sequencing																
Seq platform	Ion Torrent	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Ion Torrent	Illumina	Illumina	Illumina	Ion Torrent	Ion Torrent
Library prep/ target enrichment	Amplicon	Hybrid	Hybrid	Hybrid	Hybrid	Hybrid	Hybrid	Hybrid	Hybrid	Amplicon	Amplicon	Hybrid	Hybrid	Amplicon (single primer extension)	Amplicon	Amplicon
Sample-level minimum coverage threshold	800×	NA	30×	300×	250×	150×	300×	300×	50×	500×	125×	100×	50×	100×	500×	500×
Sample-level avg coverage for cell line exercise	1400×	1060.5×	394×	750×	982×	549×	800×	>400×	753×	>500×	314×	1517×	100×	500×	2000×	1300×
Variant-level minimum coverage	20×	50×	50×	100×	100×	50×	50×	50×	20×	100×	20×	Position-specific threshold. Determined by ML	150×	100×	60×	60×
Variant-level minimum read (ALT depth)	20	5	5	10	5	2[a]	3	3	8	10	4	6	2	4	10	10
Variant calling																
Type of variant	Non-synonymous and synonymous	Non-synonymous and synonymous	Non-synonymous only	Non-synonymous only	Non-synonymous and synonymous	Non-synonymous and synonymous	Non-synonymous and synonymous	Non-synonymous and synonymous	Non-synonymous only	Non-synonymous and synonymous	Non-synonymous only	Non-synonymous and synonymous	Non-synonymous and synonymous	Non-synonymous only	Non-synonymous only	Non-synonymous only
Germline variant filtration approach	Tumor only	Normal tissue	Tumor only	Tumor only	Tumor only	Tumor only	Tumor only	Tumor only	Normal tissue	Tumor only	Tumor only	Tumor only	Tumor only	Tumor only	Tumor only	Tumor only
Removes variants from known cancer genes	Yes	No	No	No	Yes	Yes	Yes	Yes	No	No	No	Yes	No	Yes	No	No
Published performance characteristics			30,31	32	9,20,33,34				9,35-37			38				39-42

ACT, ACT Genomics; AZ, AstraZeneca; BWH, Brigham and Women's Hospital; Caris, Caris Life Sciences; FMI, Foundation Medicine; ILLUM, Illumina; IPG, Intermountain Precision genomics; JHU, Johns Hopkins University; Mb, megabase; MSKCC, Memorial Sloan Kettering Cancer Center; NGS, next generation sequencing; PGDx, Personal Genome Diagnostics; Q2, Q Squared Solutions; Thermo_OCA, Thermo Fisher Scientific Oncomine Comprehensive Assay; Thermo_OTMLA, Thermo Fisher Scientific Oncomine Tumor Mutation Load Assay; UMI, Unique Molecular Identifier.

[a] Plus sample- and position-specific cut-off by likelihood ratio model.

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set of 29 clinical samples that were not used in any way to develop the calibration curves (testing set). For each clinical sample, the uncalibrated, TCGA-calibrated, and cell line-calibrated panel TMB values were visually compared by boxplots. Root mean squared error (RMSE), relative to the observed WES value for each sample, was calculated on the sample and on the panel assay level.

The calibration tool, tmbLab, is an open-source software package written in the publicly available statistical software R that was created as part of this study.[21] This package, vignettes, documentation, and associated source code have been made freely available for public use at https://brb.nci.nih.gov/tmbLab/. The 'tmbLab' package was applied for the calibration analyses (Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2021.09.016). Output produced by the package includes calibration plots as well as intercept, slope, and variance parameters associated with the fitted calibration curves relating panel TMB to WES TMB.

Compliance with ethics guidelines

Institutional review board (IRB) approval of the study protocol was obtained by each laboratory before study conduct. In all cases, the IRB determined this study is exempt from IRB review because it does not meet the definition of human subject research as defined in 45 CFR 46.102. Specifically, the investigators did not obtain information or biospecimens through intervention or interaction with individuals, and the DNA samples utilized by the participating laboratories was de-identified.

RESULTS

Variability across panel assays of participating laboratories

Sixteen targeted gene panel assays from academic and diagnostics laboratories participated in this study (Table 1). Each panel assay had a unique combination of characteristics that encompassed different sample processing requirements and sequencing platforms and chemistries. Each laboratory used their own analytical and bioinformatics methodologies to estimate TMB, which were optimized to their own panel assay specifications. If available, published panel assay performance characteristics are reported (Table 1). Size of the coding regions used to estimate TMB ranged from 0.8 to 1.94 Mb; minimum DNA input ranged from 20 to 150 ng and sample-level depth of coverage ranged between 30 and 800× for the participating laboratories. Seventy-five percent (12/16) of panel assays used an Illumina sequencing platform, while the others used the Thermo Fisher Scientific Ion Torrent platform. Sixty-three percent (10/16) used hybridization as a target enrichment approach, while the remaining panel assays used an amplicon-based approach.

The locally developed bioinformatics pipelines used in this phase II study also varied. All 16 panel assays included non-synonymous variants for TMB estimation, while 9 panel assays (56%) also included synonymous variants. Two panel assays used paired normal tissue to remove germline variants for TMB estimation, and the remaining 14 used their own tumor-only approach that utilized a combination of population frequency databases and proprietary methods for germline variant removal (Table 1). The variability in panel TMB values is described with boxplots in Figure 1 for the 25 clinical samples with WES TMB values <20 mut/Mb (Figure 1A and Supplementary Table S4), the 4 clinical samples with WES TMB values >20 mut/Mb (Figure 1B), and the 10 cell line samples (Figure 1C and Supplementary Table S5). Overall, the empirical variability across panel assays increased with increasing TMB value, which is consistent with findings of our previous study.[18] This trend in variance is evidenced by the wider (vertically stretched) boxplots proceeding from left to right within each figure and by comparing Figure 1A to Figure 1B. We noted that in clinical samples, WES TMB was occasionally lower than many of the reported panel TMB values (e.g. TMB-38, TMB-51, TMB-36), whereas in the cell lines, WES TMB was sometimes higher than many of the reported panel TMB values (e.g. NCI-H1437, NCI-H2009). Patient demographic and clinical variables as well as some specimen characteristics are also described via heatmaps below the boxplot figures (Figure 1).

Impact of panel assay size on panel TMB estimates

We used an *in silico* approach to estimate the impact of panel size on the PPA and NPA of TMB calling. At a TMB cut-off of 10, all 16 panel assays evaluated have a theoretical NPA of at least 95%, with a theoretical NPA falling <95% for panel sizes under 667 Kb (Figure 2A). The theoretical PPA at a TMB cut-off of 10 ranged from 87% to 92%, with a theoretical PPA falling <85% for panel sizes under 577 Kb. At a TMB cut-off of 5, theoretical NPAs ranged from 87% to 91%, while theoretical PPAs ranged from 86% to 92%, with larger panel assays having higher theoretical PPA and NPA. At TMB cut-offs of 15 and 20, theoretical NPAs ranged from 98% to 99%, while theoretical PPAs ranged from 88% to 92%.

While actual panel performance reflects many factors, including depth of sequencing and accuracy of mutation calling, we observed a substantial acceleration of decrease in PPA of panels at critical intersections of small panel sizes and low TMB cut-offs (Figure 2A). These findings support the hypothesis that small panels are insufficient to maintain adequate PPA and NPA for calling TMB high versus TMB low across the range of cut-offs for positivity likely to be used in practice.

Impact of panel assay gene content on panel TMB estimates

Failure to filter out pathogenic variants in panel TMB estimates results in overestimation of TMB relative to WES for all panel assays investigated (Figure 2B). In this *in silico* analysis, removing known pathogenic cancer gene mutations, as identified in COSMIC, showed a closer approximation to WES TMB. When synonymous variants are additionally filtered, thereby keeping only non-synonymous

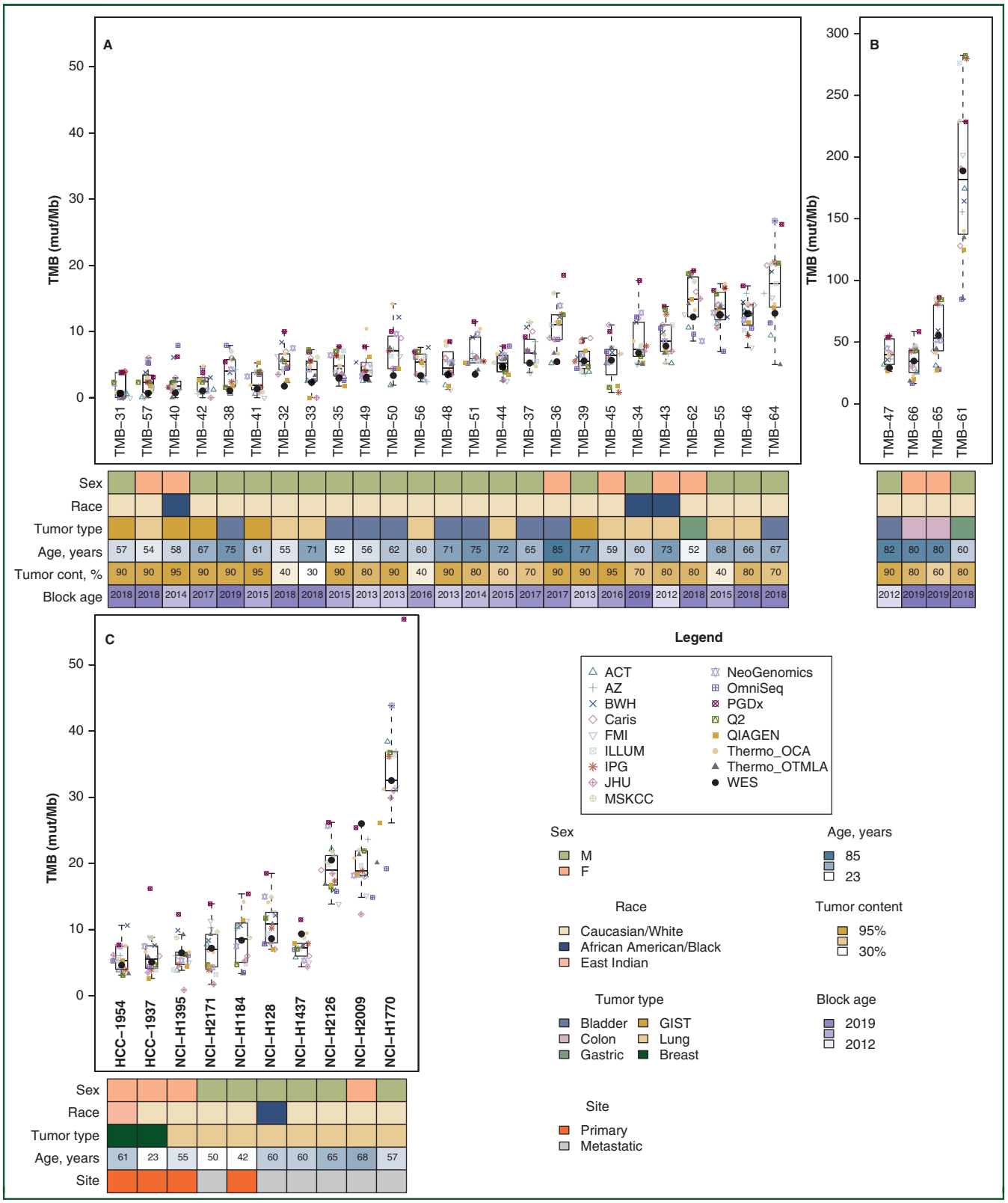

Figure 1. Variability of reported TMB values across panel assays participating in the experiment as depicted by boxplots.
Sample-level boxplots are ordered by observed WES TMB value (low to high). Heatmaps describe demographic and clinical characteristics of the sample.
(A) Clinical samples with WES TMB values <20. (B) Clinical samples with WES TMB values >20. (C) Cell lines.
ACT, ACT Genomics; AZ, AstraZeneca; BWH, Brigham and Women's Hospital; Caris, Caris Life Sciences; FMI, Foundation Medicine; GIST, gastrointestinal stromal tumor; ILLUM, Illumina; IPG, Intermountain Precision genomics; JHU, Johns Hopkins University; MSKCC, Memorial Sloan Kettering Cancer Center; PGDx, Personal Genome Diagnostics; Q2, Q squared Solutions; Thermo_OCA, Thermo Fisher Scientific Oncomine Comprehensive Assay; Thermo_OTMLA, Thermo Fisher Scientific Oncomine Tumor Mutation Load Assay; TMB, tumor mutational burden; WES, whole exome sequencing.

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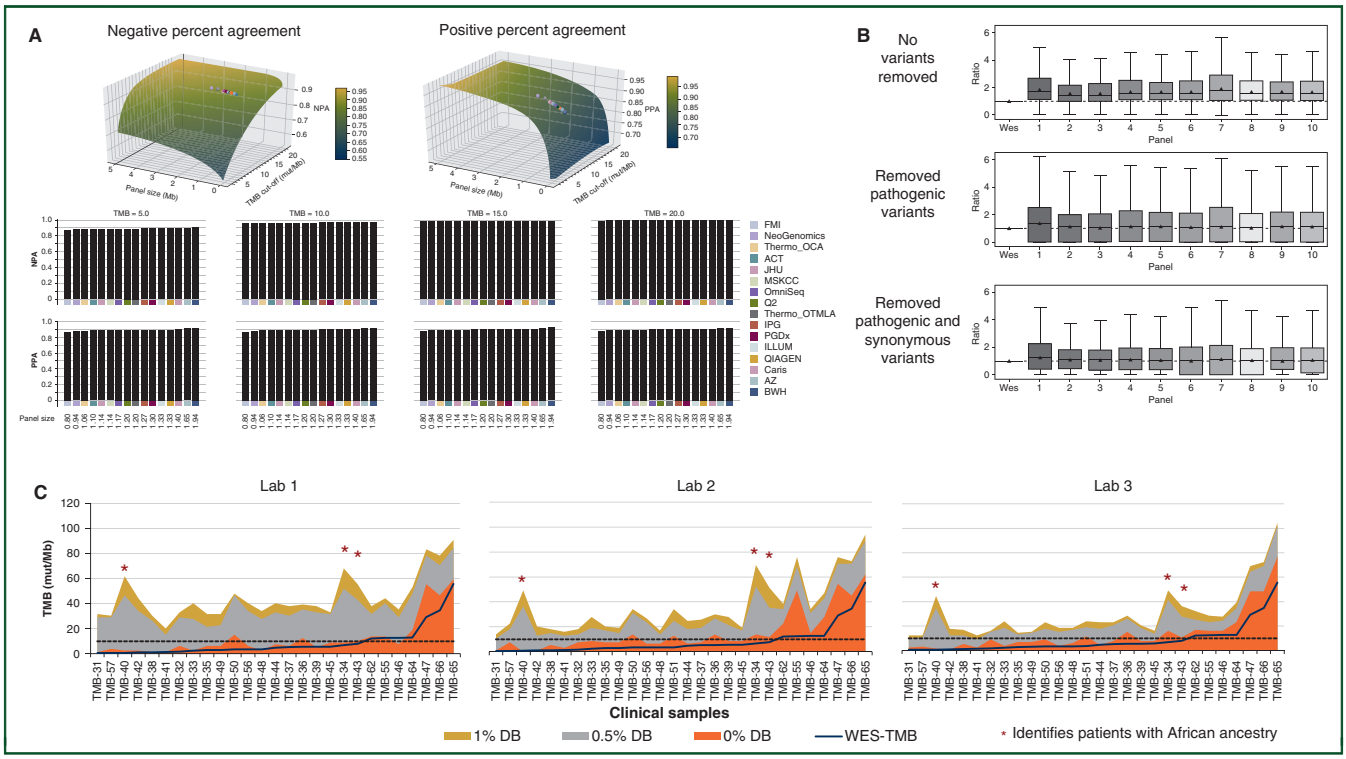

Figure 2. Panel assay design and bioinformatics factors affecting panel TMB estimates.
(A) Impact of panel assay size on NPA and PPA of panel TMB estimate, (B) impact of gene content (including pathogenic variants and synonymous variants) on panel TMB estimate, and (C) impact of population allele thresholds on germline variant filtering. *Identifies patients with African ancestry.
ACT, ACT Genomics; AZ, AstraZeneca; BWH, Brigham and Women's Hospital; Caris, Caris Life Sciences; FMI, Foundation Medicine; ILLUM, Illumina; IPG, Intermountain Precision genomics; JHU, Johns Hopkins University; MSKCC, Memorial Sloan Kettering Cancer Center; NPA, negative percent agreement; PGDx, Personal Genome Diagnostics; Q2, Q Squared Solutions; PPA, positive percent agreement; Thermo_OCA, Thermo Fisher Scientific Oncomine Comprehensive Assay; Thermo_OTMLA, Thermo Fisher Scientific Oncomine Tumor Mutation Load Assay; TMB, tumor mutational burden; WES, whole exome sequencing.

variants to estimate TMB, only a minimal effect is observed on panel TMB estimates as approximations to WES TMB. However, it was evident that removing synonymous variants also widened the boxplot, thus signaling greater variability across panel TMB estimates when the number of variants was reduced. Variability in this context was also associated with panel assay size. Boxplot width was the smallest for panel 2, which also corresponded to the largest panel assay (1.5 Mb). In contrast, panel 7, with the smallest panel assay (0.8 Mb), exhibited the greatest boxplot width.

Impact of germline variant filtering on panel TMB estimates

The tumor-only approach utilized by 14 out of 16 panel assays included the identification of common variants in a single or a combination of population-based genotyping databases (Supplementary Table S6, available at https://doi.org/10.1016/j.annonc.2021.09.016). Filtering out potential germline variant calls, defined as >0% of the pMAF, provides the strongest correlation to WES TMB independent of the panel assay utilized (Figure 2C). In some instances, use of 0% pMAF could even lead to underestimation of panel TMB. Conversely, setting the germline variant allele frequency filter to >0.5% pMAF significantly overestimates panel TMB compared to WES TMB and this effect is even more pronounced when the filter is raised to >1% pMAF. Notably, three of the clinical samples evaluated were from patients of African descent (TMB-34, TMB-40, TMB-43) and were observed to have panel TMB values that were grossly overestimated by the majority of platforms, especially if 0.5% or 1% pMAF thresholds were used for the removal of germline variants.

Calibration tool

The range of fitted calibration curve slopes across the panel assays was 0.868-1.647 when TCGA data were used as the calibration reference, and 0.551-1.142 when the cell line data were used as the reference (Supplementary Tables S7 and S8, available at https://doi.org/10.1016/j.annonc.2021.09.016). The TCGA- and cell line-derived calibration results are depicted in Figure 3 for samples with WES TMB values between 5 and 15. The boxplots of all 29 clinical samples are included in Supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2021.09.016. In general, the TCGA calibration approach tends to yield boxplots that are compressed and/or closer to the WES TMB value, when compared to uncalibrated TMB values. Numerically this is demonstrated by the lower RMSE (Supplementary Table S9, available at https://doi.org/10.1016/j.annonc.2021.09.016); in particular, the RMSE for the TCGA-calibrated TMB values as compared to the uncalibrated TMB values is equal or lower in 26/29 (90%) clinical samples (Supplementary Table S9, available at https://doi.org/10.1016/j.annonc.2021.09.016). In contrast, the cell line

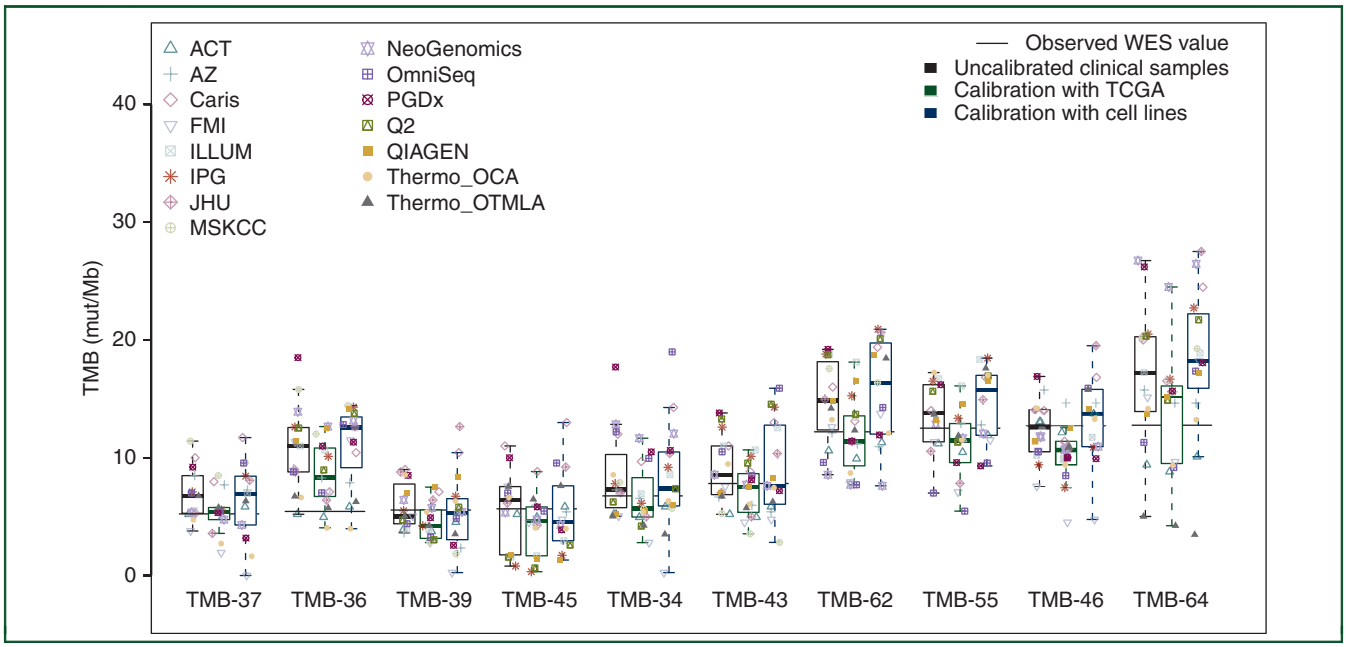

Figure 3. Application of two calibration approaches (TCGA and cell line) to the clinical samples.
Sets of boxplots are depicted for samples with WES TMB values between 5 and 15 (full sample set depicted in Supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2021.09.016). For each sample, the three boxplots correspond to (left to right) the uncalibrated TMB values, the TCGA-calibrated TMB values, and the cell line-calibrated TMB values. The black horizontal line across the three boxplots corresponds to the observed WES TMB value.
ACT, ACT Genomics; AZ, AstraZeneca; BWH, Brigham and Women's Hospital; Caris, Caris Life Sciences; FMI, Foundation Medicine; ILLUM, Illumina; IPG, Intermountain Precision genomics; JHU, Johns Hopkins University; MSKCC, Memorial Sloan Kettering Cancer Center; PGDx, Personal Genome Diagnostics; Q2, Q Squared Solutions; TCGA, The Cancer Genome Atlas; Thermo_OCA, Thermo Fisher Scientific Oncomine Comprehensive Assay; Thermo_OTMLA, Thermo Fisher Scientific Oncomine Tumor Mutation Load Assay; TMB, tumor mutational burden; WES, whole exome sequencing.

calibration approach does not yield less variable/less biased boxplots or lower RMSE than uncalibrated TMB values (Figure 3, Supplementary Figure S2 and Table S9, available at https://doi.org/10.1016/j.annonc.2021.09.016). RMSE was also calculated on the panel assay level and shows that RMSE may increase or decrease on the panel assay level (Supplementary Table S10, available at https://doi.org/10.1016/j.annonc.2021.09.016).

DISCUSSION

In an environment where diverse NGS assays will be available, to confidently use TMB estimation in clinical decision making, sources of measurement variability must be understood and controlled for when interpreting results. In this large collaboration-driven study, we describe the empirical variability in TMB estimation across 16 different panel assays applied to a common set of FFPE clinical tumor samples and to human tumor-derived cell lines. Additionally, we developed a publicly available calibration tool to align TMB estimates using different panel assays.

A certain degree of variability in the estimation of TMB on clinical samples across panel assays was expected, similar to our *in silico* assessment.[18] Factors such as panel assay content, sequencing platforms, and bioinformatics pipelines were expected to contribute to variability. Since standardization of these variables is impractical, we utilized publicly available samples to quantitatively characterize the empirical variability in panel TMB estimation and provide the opportunity to achieve more consistent results through calibration.

Our results agree with previous reports showing that a sufficiently sized panel is required to maintain reasonable PPA of panel TMB measurements.[22-26] There is a small but consistent association between panel assay size and the PPA and NPA, regardless of the TMB cut-off. However, we also found relatively marginal gains in assay performance above a certain threshold of panel size.

In addition to size alone, gene content is also a key factor. We show that filtering out known cancer gene mutations, as identified in COSMIC, significantly improved the accuracy of panel TMB estimates relative to WES TMB for all of the panel assays. Another approach is to remove synonymous alterations and count only non-synonymous variants when estimating TMB; seven participating laboratories did so in our study. However, this did not significantly affect accuracy of TMB estimates in the clinical samples (perhaps related to few silent alterations in the gene regions tested by each panel assay).[23,27]

Another issue in TMB estimation is the impact of tumor-only sequencing, which can lead to inadvertent inclusion of germline variants. Inclusion of germline variants within 1% pMAF as part of a tumor-only germline variant removal approach resulted in significant overestimation of panel TMB, which has also been observed by Parikh et al.[28] Population databases are commonly used by various panel assays, and here we showed that the most stringent filtering approach, using a filter of >0% pMAF, offers the closest approximation to WES TMB compared to other pMAF values (0.5% or 1%). Additionally, TMB values for patients of African descent within our clinical samples were overestimated. Analysis using more than one population

database may help to reduce biases, especially as databases may vary with their representation of different racial ancestries.[29] Overall, it is important to accurately filter germline variants using available bioinformatics methods. Additionally, the use of FFPE specimens may have an impact on TMB estimation by generating false positives due to artifacts created during the fixation process. These factors must be considered and assessed during assay development, including development of the bioinformatics pipeline to reduce potential false positives. Clinically, if the TMB value of a cancer is close to a predetermined threshold that would make it eligible for treatment, the variability added by the suboptimal removal of germline variants could translate to potential overtreatment of patients and unnecessary exposure to immune-related adverse events. Patient-matched normal samples are not always available to identify a patient's germline variants for filtration. Thus, it is important to accurately filter germline variants using available bioinformatics methods.

Beyond characterizing and quantifying factors that can impact variability in panel TMB estimates, we also built a tool to promote alignment and optimize the functionality of TMB as a clinical biomarker.

Our calibration tool aims to improve clinical consistency and interpretability and is a free and open-source software. Ideally, a calibration tool could be used for regulatory purposes to permit different tests to align to common treatment recommendations, resulting in expanded patient access and reduced variability in oncology care. Application of the calibration tool using TCGA data as a reference does not account for differences in wet-lab procedures across panel assays. We attempted to use human tumor-derived cell lines as a reference material; however, there were insufficient cell lines with matched normal cell lines and calibration using the 10 cell lines in this study did not meaningfully reduce variability (Figure 3). See Supplementary Table S11 for considerations for the use of different sources as reference material. More generally, the calibration tool reduced the overall variability across laboratories, but calibration did not improve concordance between panel TMB and WES TMB for every lab. Further work is needed to optimize the calibration tool for this purpose. Our findings should be interpreted considering several limitations, including the heterogeneity of tumor specimens acquired and inclusion of a few tumors for which immunotherapies are less relevant (i.e. GIST cancers) as well as use of samples with high tumor purity ($\geq$30%) which may not represent all samples acquired in the clinical setting.

Despite these limitations, our tool effectively demonstrates that calibration of panel TMB values can be achieved to an extent that supports development and utilization of TMB applications across platforms. While use of each TMB platform will likely be optimized to specific drug indications, there is value in considering the harmonization and standardization principles we present here. Based on our findings, we strongly encourage diagnostics developers to conduct their own calibration analyses and compare their panel assays to others in order to achieve optimal reproducibility and improve assay utility in the clinic. Clinicians can use findings from this study to contextualize a single TMB output. Clinically, if the TMB value of a cancer is close to a predetermined threshold that would make a patient eligible for treatment, being able to recognize variability of individual panel-level TMB values could help avoid potential over- or undertreatment of patients or unnecessary exposure to immune-related adverse events. In addition to direct clinical care, calibration may facilitate synthesizing panel TMB data across studies for translational research and enable increased scale and power of studies to examine TMB along with other predictors of response to immunotherapy.

Conclusion

The TMB Harmonization Project leveraged the expertise and insight of 16 different diagnostic laboratories to objectively evaluate the empirical variability across panel TMB values and to propose best practices for panel TMB alignment. Our work demonstrates that the utilization of a calibration tool based on a universal reference standard derived from TCGA data can enhance comparability of TMB across different panel assays. Use of different NGS platforms for TMB testing will necessitate a combinatorial approach, including consensus guidelines and availability of a universal reference standard, in order to maintain a satisfactory level of consistency in the measurement and clinical application of this complex biomarker. Availability of reference material annotated with analytical and associated clinical truth would be of value to assay development efforts. Our results provide proof of principle that this level of alignment is achievable and will support the consistent assessment, adoption, and application of TMB to optimally guide immunotherapy decisions. We hope that this process can serve as a model for future biomarker technologies and alignment efforts.

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DISCLOSURE

XZW is an employee of EMD Serono Research and Development Institute. JN, DF, and ESS are all employees of Foundation Medicine, and ESS is a shareholder in Roche. VF and LKB are employees of Neogenomics and stockholders in NeoGenomics Inc. S-JC and J-HC are employees of ACT Genomics and stockholder in ACT Genomics. JB is employed with BMS, shareholder in BMS, and a shareholder in Johnson & Johnson. JC and SP are employed by OmniSeq, Inc. and hold restricted stock in OmniSeq, Inc. DC and WT are employed with Thermo Fisher Scientific and stockholder in Thermo Fisher Scientific. KE is an employee of Intermountain Genome Diagnostics. GG is employed by BMS and a stockholder in BMS. VRG and R. Samara are employed with QIAGEN. LAK and KCV are employed with Personal Genome Diagnostics. PS is employed by Caris Life Sciences. AS serves on advisory boards and/or receives speech honoraria from AIGnostics, Bayer, Thermo Fisher, Illumina, Astra Zeneca, Novartis, Pfizer, Roche, Seattle Genetics, MSD, BMS, Takeda, Janssen, and Eli-Lily; and research funding from: Chugai and Bristol Myers Squibb. MB is employed by LGC SeraCare. VW is employed with Q Squared Solutions. JCB and MX are employed by AstraZeneca. JCB is employed and holds shares of AstraZeneca. KM and CZ are employees of Illumina Inc and stockholders in Illumina Inc. HM and GP are employees and shareholders in Biodesix Inc. MDH has stock and other ownership interests in Shattuck Labs, Immunai, and Arcus Biosciences; reports honoraria from AstraZeneca and Bristol Myers Squibb; has a consulting or advisory role with Bristol Myers Squibb, Merck, Genentech/Roche, AstraZeneca, Nektar, Syndax, Mirati Therapeutics, Shattuck Labs, Immunai, Blueprint Medicines, Achilles Therapeutics, and Arcus Biosciences; receives research funding from Bristol Myers Squibb (Inst); has patents, royalties, and other intellectual property [a patent has been filed by Memorial Sloan Kettering (PCT/US2015/062208) for the use of TMB for prediction of immunotherapy efficacy, which is licensed to Personal Genome Diagnostics]; and receives travel and accommodation expense reimbursement from AstraZeneca, Bristol Myers Squibb, and Eli Lilly. All other authors have declared no conflicts of interest.

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