High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types

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STRUCTURED ABSTRACT

Background: High tumor mutation burden (TMB-H) has been proposed as a predictive biomarker for response to immune checkpoint blockade (ICB), largely due to potential for tumor mutations to generate immunogenic neoantigens. Despite recent pan-cancer approval of ICB treatment for any TMB-H tumor, as assessed by the targeted Foundation One CDx assay in 9 tumor types, the utility of this biomarker has not been fully demonstrated across all cancers.

Patients and methods: Data from over 10,000 patient tumors included in The Cancer Genome Atlas were used to compare approaches to determine TMB and identify the correlation between predicted neoantigen load and CD8 T cells. Association of TMB with ICB treatment outcomes
was analyzed by both objective response rates (ORRs, N=1551) and overall survival (OS, N=1936).

**Results:** In cancer types where CD8 T cell levels positively correlated with neoantigen load, such as melanoma, lung, and bladder cancers, TMB-H tumors exhibited a 39.8% ORR to ICB (95% CI 34.9–44.8), which was significantly higher than that observed in low TMB (TMB-L) tumors (odds ratio (OR) = 4.1, 95% CI 2.9–5.8, P < 2×10^{-16}). In cancer types that showed no relationship between CD8 T cell levels and neoantigen load, such as breast cancer, prostate cancer, and glioma, TMB-H tumors failed to achieve a 20% ORR (ORR = 15.3%, 95% CI 9.2–23.4, P = 0.95), and also exhibited a significantly lower ORR relative to TMB-L tumors (OR = 0.46, 95% CI 0.24–0.88, P = 0.02). Bulk ORRs were not significantly different between the two categories of tumors (P = 0.10) for patient cohorts assessed. Equivalent results were obtained by analyzing OS and by treating TMB as a continuous variable.

**Conclusions:** Our analysis failed to support application of TMB-H as a biomarker for treatment with ICB in all solid cancer types. Further tumor type specific studies are warranted.

**Keywords**
immune checkpoint blockade; tumor mutation burden; biomarker

**INTRODUCTION**

The advent of immune checkpoint blockade (ICB) targeting programmed cell death protein 1 (PD-1) and the programmed death-ligand 1 (PD-L1) proteins have revolutionized cancer therapy, providing robust, durable responses in a subset of patients with cancer. High tumor mutation burden (TMB-H) is a leading candidate biomarker for identifying patients with cancer who may benefit from ICB based on the underlying assumption that increasing the numbers of mutant proteins will create antigenic peptides allowing for enhanced immunogenicity\(^1\)\(^{-4}\). Accumulating evidence supports this hypothesis in certain cancers that are generally more hypermutated\(^5\), with the majority of evidence for improved ICB response in TMB-H tumors coming from lung cancer and melanoma datasets\(^2\).

In 2017, the U.S. Food and Drug Administration (FDA) approved pembrolizumab (anti-PD-1) for treatment of unresectable or metastatic tumors from any tumor histology that exhibits microsatellite instability (MSI) and/or DNA mismatch repair deficiency (dMMR), resulting in a hypermutator phenotype, marking the first tumor type agnostic predictive biomarker approval\(^6\). Approved testing methods include: MMR protein immunohistochemistry, PCR for MSI, and/or next-generation sequencing. TMB-H has been evaluated as a predictive biomarker in numerous ICB studies over recent years; however, diverse methods have been used to quantify TMB with different thresholds for what is considered TMB-H.

Recently, the FDA also approved pembrolizumab for the treatment of patients with any unresectable or metastatic non-dMMR/MSI TMB-H tumor that has progressed on prior therapy with no alternative treatment options, which has been met with mixed responses from the scientific community\(^7\),\(^8\). In the approval, TMB-H was defined as ≥10 mutations/
megabase of DNA (mut/Mb), as determined by the targeted sequencing FoundationOne CDx (F1CDx) assay, which profiles the total number of synonymous and non-synonymous mutations across 324 cancer-related genes. The FDA approval was based on ORR data from 102 patients, of which 81 were MSS, tumors across 9 cancer types treated in the phase 2 KEYNOTE-158 study, with the plurality (33%) of tumors coming from small cell lung cancer\(^9,10\). The trial lacked major tumor types for which approval for PD1-blockade is lacking, including estrogen receptor positive breast cancer, prostate cancer, and MSS colorectal cancer, which are the most common newly diagnosed cancer in women, the most common newly diagnosed cancer in men, and the third most common newly diagnosed cancer for both sexes, respectively\(^11\).

While evidence does support treatment of certain hypermutator cancer subsets with ICB, such as successes in patients with MSI-H colorectal tumors\(^12,13\), it is unclear if this principal is generalizable across cancer types, and what is an optimal threshold, if any, for qualifying a cancer as TMB-H for ICB-treatment selection\(^2,7,14–16\). Furthermore, accumulating evidence indicates that TMB assessment and bioinformatics interpretation varies across different targeted sequencing panels, likely in a cancer-type dependent manner\(^17\). In this work, we first demonstrate classification of tumors as TMB-H varies in a cancer type-specific manner across different DNA sequencing assays/approaches in over 10,000 patients spanning 31 cancer types from The Cancer Genome Atlas (TCGA). We further leverage TCGA data to divide cancers into two categories based on whether CD8 T cell infiltration is positively correlated with neoantigen load. Next, we show that TMB-H, as defined in the recent FDA-approval as 10 mut/Mb determined in the context of F1CDx assay genes or when treated as a continuous variable, is suboptimal for predicting objective response and overall survival following ICB treatment across multiple cancer types where neoantigen load is not associated with CD8 T cell infiltration. Notably, TMB-H failed to show predictive accuracy for ICB response in patients with glioma, prostate cancer, breast cancer, and other tumor types where a biomarker to optimize patient selection is most urgently needed due to a lack of broad ICB approval. Together, these data urge strong caution in using TMB as a predictive biomarker for ICB therapy in a pan-cancer fashion across all solid cancer types.

**METHODS-PATIENTS**

**ARTEMIS TNBC cohort description and whole exome sequencing.**

The ARTEMIS cohort consists of patients with TNBC who did not respond to 4 cycles of neoadjuvant Adriamycin/cyclophosphamide. Patients were then stratified to one of four molecularly targeted therapy arms or physician’s choice control arm\(^17\). Patients demonstrating high levels of lymphocyte infiltration were stratified to receive combination anti-PD-L1 and nab-paclitaxel. For whole exome sequencing, DNA isolated from fresh-frozen tumor biopsies and matched peripheral blood cells was sheared by sonication prior to library preparation using the KAPA library prep kit (KAPA). Exome capture was performed with Roche Nimblegen (Exome V3) kit, followed by sequencing on a HiSeq 2000 (Illumina Inc., San Diego, CA, USA) using a version 3 TruSeq paired end flowcell. DNA quality/fragment size was confirmed by DNA Tape for the 2200 Tapestation (Agilent) throughout the preparation. Following demultiplexing with CASAVA 1.8.2, we called single nucleotide
variants (SNVs) using our previously described pipeline\textsuperscript{37} implemented in the BETSY system\textsuperscript{38}. Briefly, after adapter trimming with Trimmomatic, alignment with BWA\textsuperscript{39}, and pre-processing including flagging duplicated reads, indel realignments, and base recalibration using Picard\textsuperscript{40} and GATK\textsuperscript{41}, variants were called using a consensus of six mutation callers. Mutations were filtered for support by at least 20 reads and 5\% variant allele frequency, and then annotated by Annovar\textsuperscript{42} and SnpEff\textsuperscript{43}. TMB was calculated as the total number of SNVs and indels divided by the 44 Mb of DNA sequenced.

**HNSCC cohort description.**

Patients with HNSCC underwent clinical sequencing and signed a clinical consent permitting results used for further research on an IRB-approved research protocol. Patients with HNSCC treated with anti-PD1 who had objective response rates were retrospectively compiled for analysis. Of 23 patients, 8 were never smokers and 13 had received one more prior lines of therapy.

**Patient cohorts from literature.**

Cohorts of patients treated with immune checkpoint blockade are compiled in Table S1, along with treatment modality and outcome analyzed. TCGA data were acquired from the pan-cancer atlas release (https://gdc.cancer.gov/about-data/publications/pancanatlas)\textsuperscript{47}.

**METHODS-ETC**

**Correlation of neoantigen load and CD8 T cell levels.**

Data for neoantigen loads and CD8 T cell infiltrates are described in Thorsson et al.\textsuperscript{44}. In brief, RNAseq samples were used to determine patient HLA types, and then used to determine neoantigens in conjunction with patient-specific mutations using NetMHCpan\textsuperscript{45}. Final neoantigen predictions were filtered for binding affinity (IC\textsubscript{50} < 500 nM) and expression levels (>1.6 transcripts per million). CD8 T cells were estimated using CIBERSORT\textsuperscript{46}. Final correlation between neoantigen load and CD8 T cells and neoantigen load was assessed by Spearman correlation coefficient. Category I cancer types were defined as those with a significant (P<0.05) positive Spearman correlation coefficient, and all other cancer types were considered Category II.

**In silico comparison of whole exome and targeted sequencing approaches.**

TCGA mutation calls were acquired from MC3 calls used in the pan-cancer atlas (https://gdc.cancer.gov/about-data/publications/pancanatlas)\textsuperscript{47}. For all analyses, a threshold variant allele frequency (VAF) of 0.05 was used as minimum value to count a mutation. To generate a F1CDx TMB, all synonymous and non-synonymous mutations in genes included in the F1CDx panel were summed and normalized by total length, consistent with the calculation used in the F1CDx assay. To generate a MSKCC IMPACT TMB, all non-synonymous mutations in genes included in the IMPACT panel were summed and normalized by total length. To generate the whole exome based TMB, all non-synonymous mutations in genes were summed and normalized by total length. Targeted sequencing panel annotations were acquired from OncoKB (https://www.oncokb.org/cancerGenes). To analyze concordance between TMB-H (>10 mut/Mb) from the F1CDx assay and other approaches, we assessed...
the ability of WXS or IMPACT TMB to identify TMB-H tumors by area under the receiver operator characteristic (AUROC) curve. Optimal WXS or IMPACT TMB to determine TMB-H by F1CDx was determined by Youden’s J statistic. Confidence intervals were determined by bootstrapping.

Statistics and data analysis.

Cohorts of patients analyzed are described in Table S1. Groups were bifurcated into TMB-H and TMB-L based on a threshold corresponding to the 10 mut/Mb value achieved by the F1CDx assay. MSI-H tumors in all cohorts were excluded from analysis for consistency with the FDA approval. For comparing objective response rates between two groups, significance was assessed with Fisher’s exact test. For testing if response rates exceeded 20%, a binomial test was used. Confidence intervals for response rates with binary categories were assessed using the Clopper-Pearson method. For comparison of response rates across multiple cohorts with binary categories, odds ratio and significance were determined by the Cochran-Mantel-Haenszel method. Analysis of the relationship between TMB (log-transformed) as a continuous variable and ICB response rates was performed using logistic regression, taking cohort as a random effect. To test survival between two groups, a log-rank test was used. For merged survival analysis across multiple cohorts/cancer types, a Cox proportional hazards model was used with cohort/cancer types used as a stratification variable.

RESULTS

Primary considerations for TMB-H as a universal ICB biomarker.

The TMB-H biomarker is predicated on the concept that increased mutational load will correspond with more immunogenic neoantigens. However, we recently demonstrated that many cancer types, such as breast and prostate cancers, do not exhibit a positive correlation between CD8 T cell infiltration and neoantigen load. Repeating this analysis with updated TCGA data from the pan-cancer atlas produced similar trends (Figure 1A). We found that only roughly a quarter of new cancer cases were in types where there was a positive correlation between neoantigen load and CD8 T cell infiltration (Category I cancers), whereas over 50% of estimated new cancer cases come from cancer types where neoantigen load does not correlate with CD8 T cell infiltration (Category II cancers) (Figure 1B). The 9 tumor types contained within the KEYNOTE158 study (Figure S1) accounted for only 11% of estimated new cases (Figure 1B).

The F1CDx assay, approved to classify tumors as TMB-H at a threshold value of ≥10 mut/Mb, profiles the total number of synonymous and non-synonymous mutations across the coding regions of 324 cancer-associated genes. We sought to analyze the accuracy of this TMB-H approach to predict response to ICB across cancer types, stratified by CD8 T cell infiltration correlation with neoantigen load. However, TMB estimates can vary based on sequencing approach, in particular when using targeted sequencing panels, potentially confounding analysis when merging multiple independent cohorts. To address this, we sought to leverage WES data from TCGA for in silico analysis to determine the TMB threshold that best identified TMB-H as assessed by the genes included in the F1CDx assay. To assess the feasibility of this approach, we utilized data from one of the most common
malignancies studied, non-small cell lung cancer (NSCLC), with paired WES and targeted-panel IMPACT data, as paired F1CDx and WES data are not available for analysis. In the TCGA lung adenocarcinoma (LUAD) cohort, we found that WES TMB robustly predicted TMB-H (≥10 mut/Mb) by IMPACT genes [area under the receiver operator characteristic (AUROC) 0.966] and, based on Youden’s J statistic, identified that a WES TMB of 7.31 would optimally identify TMB-H LUAD tumors assessed by IMPACT genes (Figure S2A). Utilizing this threshold in a cohort of lung tumors sequenced by both IMPACT and WES, we found that while using a 10 mut/Mb WES threshold only recovered 54% of TMB-H tumors as assessed by IMPACT targeted sequencing, our modified threshold was able to recover over 80% of TMB-H tumors (Figure S2B), indicating this approach may be useful for unifying datasets across multiple sequencing platforms. Both thresholds were 100% specific (Figures S2C). Applying this approach to the F1CDx assay genes instead, we again found TMB from WES accurately identified TMB-H tumors from F1CDx genes, with a mean AUROC curve of 0.98 (range 0.82–1.00, Figure S2D). However, the WES TMB value that optimally identified TMB-H tumors determined from F1CDx genes varied by cancer type (range 0.8–13.8 mut/Mb), with the use of F1CDx genes leading to overestimation of TMB in 25 out of 31 cancer types analyzed (Figure S2E), consistent with prior approaches to harmonize TMB across assays. Overall, there was a strong negative correlation between the median TMB in a given cancer type and the degree to which the TMB-H threshold was overestimated (Figure S2F).

**TMB-H predicts response to ICB in Category I cancer types where neoantigen load correlates with CD8 T cell levels.**

Using the F1CDx threshold value for TMB-H to unify external datasets, we sought to analyze if TMB-H was associated with improved response to ICB across cancer types. As TMB and neoantigen load have been shown to be strongly correlated, we hypothesized that TMB-H tumors would exhibit improved ICB response rates in Category I cancer types where neoantigen load correlated with increasing CD8 T cell infiltration in our analysis of TCGA tumors (Figure 1A). Endometrial cancer from the KEYNOTE158 trial demonstrated a significantly higher response rate in TMB-H tumors compared to TMB-L tumors (Figure 2A), and the TMB-H cervical cancer tumors from the KEYNOTE158 trial showed a trend towards better response rates (Figure 2B). TMB-H colorectal cancer showed a trend towards increased response rate in a merged cohort of patients (Figure 2C), and a similar trend was observed when analyzing data Chalabi et al. alone (Figure S3B). TMB-H melanoma also demonstrated significantly higher ICB response rates in 2 out 3 independent cohorts, and all 3 TMB-H melanoma cohorts showed response rates significantly greater than 20% (Figure 2D–F). Similar trends were observed in 3 cohorts of patients with bladder cancer treated with ICB (Figure 2G–I), and 4 cohorts of patients with NSCLC adenocarcinomas (Figure 2J–M). When analyzing OS data, we found that a significantly improved prognosis for TMB-H colorectal cancer (P = 0.01) and melanoma (P = 5.3×10⁻⁴), with trends toward improved prognosis in TMB-H NSCLC adenocarcinomas and bladder cancer (Figure 2N, S3A).
TMB-H does not predict response to ICB in Category II cancer types where neoantigen load is not positively correlated with CD8 T cell levels.

We next sought to evaluate the predictive ability of TMB-H in Category II cancer types where CD8 T cell levels are not associated with neoantigen load. Patients with TMB-H anal cancer exhibited amongst the lowest response rates within the KEYNOTE158 trial, with a trend towards worse response rates relative to TMB-L tumors (Figure 3A). In gastric cancer, no difference in response rate was observed between TMB-H (16.7%) and TMB-L (16.2%) tumors treated with ICB (Figure 3B). A similar trend was observed in patients with head and neck squamous cell carcinoma (HNSCC, Figure 3C), which we validated in a newly generated internal cohort (Figure 3D). Comparable results were observed in patients with squamous lung cancer (Figure 3E–F) and various mixed squamous cell carcinomas (Figure 3G). TMB-H clear cell renal cell carcinoma (ccRCC) also exhibited a lower response rate to ICB than TMB-L tumors (Figure 3H). In ccRCC treated with everolimus, TMB-H tumors exhibited a higher response rate compared to TMB-L tumors, indicating that the lower response rate to ICB does not represent intrinsic therapeutic resistance in TMB-H tumors (Figure S3C). In patients with metastatic MSS triple negative breast cancer (TNBC)\(^24\), no objective responses occurred in 10 total TMB-H tumors, compared to a 20.5% response rate in TMB-L tumors (Figure 3I). In the ARTEMIS trial, patients with TNBC that did not achieve substantial volumetric reduction after the first phase of neoadjuvant therapy with adriamycin/cyclophosphamide were recommended to enroll in one of four molecularly targeted therapy trials in combination with nab-paclitaxel as the second phase of their neoadjuvant treatment\(^25\). Patients with TNBC that had stromal tumor infiltrating lymphocytes ≥10% were generally recommended to receive combination anti-PD-L1 and nab-paclitaxel. Of patients with TMB-H tumors, an equivalent response rate (pathologic complete response or minimal residual disease at the time of surgical resection) was observed in patients who received ICB compared to other targeted therapies (Figure 3J). In patients with prostate cancer treated with ICB (anti-CTLA4), although no objective responses were observed in the entire cohort, the percentage of patients who achieved clinical benefit was slightly higher in TMB-L tumors (36.8%) relative to TMB-H tumors (25.0%) (Figure 3K). While attempting to optimize TMB threshold by minimizing the P-value determined by Fisher’s exact test did improve TMB-H response rates and significance for lung, bladder, and melanoma cohorts, the optimal threshold varied across cancer types (Figure S4A). Moreover, the response rates in TNBC, ccRCC, and SCC patients with TMB-H tumors remained lower than TMB-L tumors (Figure S4B).

Analysis of overall survival in ICB-treated patients with prostate cancer also found TMB-H tumors exhibited numerically worse outcomes compared to those with TMB-L tumors (Figure 3L). Further analysis of overall survival in a selection of Category II cancers treated with ICB revealed trends towards worse outcomes in TMB-H esophageal cancer, squamous NSCLC, breast cancer, glioma, and ccRCC (Figure 3M, S3A). Upon comparing overall survival following treatment with ICB compared to other therapeutic modalities in only patients with TMB-H tumors, we found that patients with TMB-H breast tumors treated with ICB exhibited worse outcomes than those treated with other modalities (Figure 3N). Worse overall survival following ICB treatment was also observed in patients with TMB-H glioma compared to other therapeutic regimens (P = 2.3×10\(^{-5}\), Figure 3O). The data in Figure 3L–O

Ann Oncol. Author manuscript; available in PMC 2021 May 01.
did not arise from randomized trials and could have other underlying confounding variables contributing to the marked difference in overall survival.

**TMB-H does not predict response to ICB in all cancer types.**

Although the relationship between TMB and ICB response in each individual cohort may be informative, these analyses are fundamentally plagued by limited power due to small sample sizes. Based on response rates across all patients in the 24 cohorts analyzed here, only the bladder cancer cohort from Mariathasan et al.\textsuperscript{26} achieved over 80% power to detect a significantly different ICB response rate between TMB-L and TMB-H tumors. The cohort with the second highest number of samples (Braun et al.\textsuperscript{27}) only achieved 56% power. To address this, we pooled all cohorts from Category I cancer types where neoantigens positively correlated with CD8 T cell levels, and those from Category II cancer types where CD8 T cell levels were not positively correlated with neoantigen levels, resulting in over 99% power to detect an increased ICB response rate in TMB-H tumors for both cancer categories.

In Category I cancer types, where neoantigen levels correlated with CD8 T cells, TMB-H tumors showed significantly higher response rates to ICB compared to TMB-L tumors (Cochran-Mantel-Haenszel \(P = 1.9\times10^{-13}\), \(OR = 3.63\), 95% CI 2.55–5.16, Figure 4A). In contrast, for breast, prostate, and other Category II cancer types, where CD8 T cell infiltration was not associated with neoantigen load, we found that not only did TMB-H tumors not exhibit a higher response rate than TMB-L tumors, but that TMB-H tumors exhibited a significantly lower response rate (Cochran-Mantel-Haenszel \(P = 0.02\), \(OR = 0.46\), 95% CI 0.24–0.88, Figure 4B). Category II cancers also failed to achieve a 20% ORR (\(P = 0.95\), Figure 4C). No significant difference in ORR was observed between Category I and II cancer types (Figure S5). Receiver-operating characteristic (ROC) curves for the ability of TMB to predict ICB response across varying thresholds further indicated that no alternative TMB threshold would accurately identify ICB responders in Category II cancers from data sets analyzed (Figure S6). Quantification of area under ROC curves (AUROC) demonstrated that TMB predicted response significantly better than random chance (Wilcoxon sign-rank test P-value = 2.0×10\(-3\)) for Category I cancers, but not Category II cancers (\(P = 0.95\), Figure 4D). Integrated logistic regression of all cohorts likewise found that TMB as a continuous variable was significantly associated with ICB response in Category I (\(P = 2.4\times10^{-11}\)), but not Category II (\(P = 0.70\)) cancers (Figure 4E). Analysis of overall survival following ICB treatment indicated that as a whole while TMB-H was associated with improved prognosis in Category I cancers (\(P = 9.8\times10^{-7}\)), whereas in Category II cancers TMB-H tumors exhibited worse overall survival compared to TMB-L tumors (\(P = 0.01\)) (Figure 4F). Similar results were obtained using TMB as a continuous variable (Figure 4G). Taken together, these data do not support utilization of TMB as a biomarker for ICB therapy in all cancer types.

**DISCUSSION**

While TMB-H demonstrates promise as a predictive biomarker for patient selection for ICB treatment, our analysis fails to support the hypothesis that a single TMB threshold can
identify patients in a pan-cancer fashion who may benefit from ICB. In particular, we find that TMB-H, compared to TMB-L, indicates neither an improved response rate nor a response rate exceeding 20% for certain cancer types which do not demonstrate a correlation between neoantigen load and CD8 T cell infiltration, such as breast and prostate cancers.

While this study is limited by retrospective analyses across various DNA sequencing approaches (with minimal data using F1CDx directly), variations in immune checkpoint inhibitors utilized, and many cohorts only consisting of objective response data instead of overall survival outcomes, the preponderance of the evidence fails to support universal treatment of TMB-H cancers with ICB. In order to better implement TMB-H as a robust clinical biomarker, critical challenges focused on both accurately determining TMB in a given cancer type and then determining an optimal TMB threshold (if one exists) must be addressed.

The current most accurate approach to determine TMB is whole exome sequencing of paired tumor and normal tissues, although this approach is both cost and time prohibitive in a clinical setting\textsuperscript{17}. To address this, targeted sequencing assays enriched for known cancer-driving gene mutations are used to assess TMB, including implementation of the F1CDx assay that was recently approved as a companion diagnostic by the FDA to assess TMB in solid tumors\textsuperscript{9}. In addition to technical variation and biological variation arising from intratumor heterogeneity\textsuperscript{28}, enrichment for common known cancer driver genes stands to inflate TMB values determined by targeted sequencing assays, leading to active research in approaches to harmonize TMB across assays\textsuperscript{17,19,20}. For instance, when comparing TMB determined by the targeted IMPACT panel versus WES, over 90% of tumors exhibited higher TMB values by the targeted IMPACT panel\textsuperscript{29}. Zehir et al. also found the increased sequencing depth achieved by targeted sequencing offered more sensitive detection of mutations, which would further increase TMB beyond what could be modeled through \textit{in silico} analysis of WES data\textsuperscript{29}. Inflation due to enrichment of driver genes in targeted sequencing panels is further amplified in lower mutation burden cancers, where driver mutations make up a larger fraction of the total number of mutations (Figure S2). Moreover, in contrast to typical WES approaches, some targeted panels, including the FDA approved F1CDx assay, do not use a paired normal sample to account for germline mutations, instead opting for bioinformatic approaches as an attempt to remove germline variants. Inclusion of potential germline variants may further exacerbate TMB inflation associated with targeted sequencing assays. Additional factors including inclusion of synonymous mutations and minimum variant allele frequency required for a variant to count as a mutation need to be accounted for when comparing TMB derived from these various platforms. The cancer-type specific variability between gold-standard TMB derived from WES versus targeted-panel sequencing requires further optimization. Potential solutions include exclusion of hotspot driver mutations from TMB calculations, inclusion of additional non-driver genes in targeted sequencing panels based on their association with TMB, cancer-type specific definitions for TMB-H, or a combination thereof.

Following optimization of the technical aspects of determining which tumors are TMB-H, additional studies would be required to robustly validate the utility of a one-size-fits-all TMB-H predictive biomarker for ICB response. We found that cancer types which show no correlation between neoantigen load and CD8 T cell infiltration not only fail to achieve a
20% response rate to ICB in TMB-H tumors, but TMB-H tumors may even demonstrate a worse response rate to ICB than TMB-L tumors. A potential explanation is that ICB sensitivity to largely driven by presence of pre-existing CD8 T cells, and that the predictive accuracy of TMB-H in certain cohorts is predominately a reflection of high basal immune cell infiltration. Notably, TMB-H did not predict ICB response in both breast and prostate cancer cohorts analyzed, the most prevalent cancers in women and men, respectively\textsuperscript{11}. Moreover, neither breast nor prostate cancers were included in the trials leading to FDA approval of ICB for TMB-H (defined as 10mut/Mb by the F1CDx assay) tumors. Beyond breast and prostate cancer, we additionally found no support for the use of ICB in TMB-H glioma. A prior phase II trial found no responses to ICB out of 13 patients with MSI-H glioblastoma\textsuperscript{30}, and other studies have found that extremely low TMB was associated with glioblastoma immunotherapy response\textsuperscript{31}, further suggesting TMB-H may not be sufficient for assessing glioblastoma sensitivity to ICB. Our observation of worse overall survival in TMB-H glioma patients treated with ICB compared to other therapeutic modalities was also observed in an independent study of glioma\textsuperscript{32}, urging extreme caution when considering TMB-H as an ICB biomarker in glioma. The particularly poor outcomes observed with ICB treatment in TMB-H glioma may be partially due to the unique immune microenvironment enriched for activated microglia and monocytes in gliomas compared to other solid tumors and even brain metastases\textsuperscript{33}. Additional complications may arise from chemotherapy-induced mutations. It has been shown that clonal neoantigens provide a better indicator of sensitivity to ICB\textsuperscript{34}, whereas chemotherapy-induced mutations will predominantly be subclonal\textsuperscript{35}. The concern of chemotherapy-induced mutations may be particularly relevant in glioblastoma, where tumor recurrence is often associated with high levels of subclonal temozolomide mutations\textsuperscript{36}. As using correlation between neoantigen load and CD8 T cell levels to identify tumor types where TMB-H may serve as a biomarker predictive biomarker for ICB response is likely suboptimal; future studies further exploring which tumor types TMB-H predicts response to ICB are warranted.

Thus, taken together current evidence fails to support the use of TMB-H as a biomarker for ICB treatment in all tumor types, including the FDA approved threshold of 10 mutations/Mb. Future studies should focus both on improving cancer type-specific assessment of TMB from targeted sequencing and cancer type-specific activity of ICB in TMB-H tumors prior to broad clinical implementation.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**FUNDING**

D.J.M. was supported by NCI grant K99CA240689. This work was supported by NCI grant R01 CA218287 and a George and Barbara Bush Endowment for Innovative Cancer Research to S.-Y.L. P.G.P. was supported by a Young Investigator Award from the Kidney Cancer Association and Prostate Cancer Foundation Young Investigator Award. Additional funding was provided by the MD Anderson Breast Cancer Moonshot program, as well as CPRIT grants RP170668 to J.T.C. and RP160710 to J.T.C. and S.L.M.
DISCLOSURE

D.J.M., P.G.P., E.J., and S.Y.L have a pending patent on a gene expression signature to predict response to immune checkpoint blockade. M.K. has served as a consultant or advisory roles for Janssen, AbbVie, Ipsen, Pfizer, Roche, and Jackson Laboratory for Genomic Medicine and received research funding from AbbVie, Bristol-Myers Squibb (BMS), and Specialized Therapeutics. A.B.H. has stock options and is an advisory board member of Caris Life Sciences. A.B.H. also serves on the advisory board of WCG Oncology, has received licensing fees from Celldex Therapeutics and DNAtrix and received research funding from Merck. M.K. has advisory role for BMS, Roche, MSD and Daichi Sankyo, and the institute receives research funding from AstraZeneca, BMS and Roche outside the submitted work. All remaining authors have declared no conflicts of interest.

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HIGHLIGHTS

• High tumor mutation burden (TMB-H) failed to predict improved or clinically relevant response to immune checkpoint blockade in all cancer types.

• Cancer types where TMB-H does not predict response generally show no relationship between tumor neoantigen load and CD8 T cell infiltration.

• Further studies should be performed before application of TMB-H as a biomarker for ICB in all cancer types.
Figure 1. Most new cases of cancer in the U.S. arise from tumor types where CD8 T cell infiltration is not associated with neoantigen load.

(A) Spearman correlation between CD8 T cell infiltration determined from RNAseq and neoantigen load across cancer types identifies two Categories of tumor types, those where increasing neoantigen load significantly correlates with increased CD8 T cell infiltration (Category I, red), and those where increasing neoantigen does not correspond with increased CD8 T cell infiltration (Category II, blue). Example correlation plots are shown to right.

(B) Common types of newly diagnosed cancer in the U.S., representing over 80% of solid tumors\textsuperscript{11,48}. Red bars indicate Category I cancers where CD8 T cell levels positively correlate with neoantigen load and blue bars indicate Category II cancers where CD8 T cells are not associated with neoantigen load. Inset percentages indicate estimated percent of new cases included in FDA Approval (11%).
cases. Gray bars indicate tumor types that could not be classified due to lack of samples in TCGA. # indicates tumor types included in FDA approval.

ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LAML, Acute Myeloid Leukemia; LGG, Brain Lower Grade Glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; OV, Ovarian serous cystadenocarcinoma; NSCLC, Non-small cell lung cancer; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SCLC, Small cell lung cancer; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumors; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma; UVM, Uveal Melanoma
Figure 2. High TMB predicts ICB response in Category I tumors where CD8 T cells positively correlate with neoantigen load.

(A) ICB response rate in metastatic endometrial cancer from KEYNOTE158 stratified by TMB.
(B) ICB response rate in metastatic cervical cancer from KEYNOTE158 stratified by TMB.
(C) ICB response rate in microsatellite stable (MSS) colorectal cancer from Chalabi et al. (localized) and Goodman et al. (metastatic) stratified by TMB. Cochran-Mantel-Haenszel.
(D) ICB response rate in metastatic melanoma from Goodman et al. stratified by TMB.
(E) ICB response rate in metastatic melanoma from Hugo et al. stratified by TMB.
(F) ICB response rate in metastatic melanoma from Miao et al. stratified by TMB.
(G) ICB response rate in metastatic bladder from Mariathasan et al. stratified by TMB.
(H) ICB response rate in metastatic bladder from Snyder et al. stratified by TMB.
(I) ICB response rate in metastatic bladder from Miao et al. stratified by TMB.

(J) ICB response rate in metastatic non-small cell lung cancer (NSCLC) adenocarcinomas from Goodman et al. stratified by TMB.

(K) ICB response rate in NSCLC adenocarcinoma from Rizvi et al. (JCO) stratified by TMB.

(L) ICB response rate in metastatic NSCLC adenocarcinoma from Rizvi et al. (Science) stratified by TMB.

(M) ICB response rate in metastatic NSCLC adenocarcinoma from Hellmann et al. stratified by TMB.

(N) Hazard ratio for overall survival following ICB treatment stratified by TMB in various cancer types from Samstein et al., with negative $\log_2$(Hazard Ratio) representing better outcomes in patients with TMB-H tumors.

Comparisons of response rates made using Fisher’s exact test unless otherwise specified, inset value indicates (# of responses/total # of cases). Survival comparisons made using log-rank test.
Figure 3. High TMB does not predict ICB response in Category II tumors where neoantigen load is not associated with increased CD8 T cell levels.

(A) ICB response rate in metastatic anal cancer from KEYNOTE158 stratified by TMB.
(B) ICB response rate in MSS metastatic gastric cancer from Kim et al. stratified by TMB.
(C) ICB response rate in metastatic head and neck squamous cell carcinoma (HNSCC) from Goodman et al. and Miao et al., stratified by TMB.
(D) ICB response rate in HNSCC from MDACC patients stratified by TMB.
(E) ICB response rate in lung squamous cell carcinomas from Hellmann et al. stratified by TMB.
(F) ICB response rate in lung squamous cell carcinomas from Goodman, Rizvi, and Miao cohorts stratified by TMB.
(G) ICB response rate in mixed metastatic squamous cell carcinoma (SCC) (head & neck, lung, urethral, cervical, and unknown) from Goodman et al. stratified by TMB.

(H) ICB response rate in metastatic clear cell renal cell carcinoma from Braun et al. stratified by TMB.

(I) ICB response rate in metastatic triple negative breast cancer from Voorwerk et al. stratified by TMB.

(J) ICB response rate in adriamycin/cyclophosphamide-resistant TMB-H TNBC from ARTEMIS trial treated with either ICB compared to other targeted therapeutics.

(K) ICB clinical benefit rate in metastatic prostate cancer from Subudhi et al. stratified by TMB. No objective responses were observed.

(L) Kaplan-Meier curve showing overall survival following ICB treatment stratified by TMB in metastatic prostate cancer from Subudhi et al.

(M) Hazard ratio for overall survival following ICB treatment stratified by TMB in various cancer types from Samstein et al., with negative log\(_2\) (Hazard Ratio) representing better outcomes in patients with TMB-H tumors.

(N) Hazard ratio for overall survival following ICB or non-ICB treatment stratified in high-TMB metastatic breast cancer from Samstein et al. \(N = 259\).

(O) Hazard ratio for overall survival following ICB or non-ICB treatment stratified in high-TMB glioma from Samstein et al. \(N = 207\).

Comparisons of response rates made using Fisher’s exact test, inset value indicates (# of responses/total # of cases). Survival comparisons made using log-rank test.
Figure 4. High TMB does not predict ICB response across all cancer types.
(A) ICB response rate in all Category I cohorts where neoantigen load correlates with CD8 T cell levels from Figure 2. Inset numbers indicate (number of responders / total number), error bars indicate 95% confidence interval. Odds ratio and significance determined by the Cochran-Mantel-Haenszel method.
(B) ICB response rate in all Category II cohorts where neoantigen load does positively not correlate with CD8 T cell levels from Figure 3. Inset numbers indicate (number of responders / total number), error bars indicate 95% confidence interval. Odds ratio and significance determined by the Cochran-Mantel-Haenszel method.
(C) ICB response rate in all merged TMB-H cohorts. Inset numbers indicate (number of responders / total number), error bars indicate 95% confidence interval. P-values over bars are for alternative hypothesis that the response rate is different than 20%. Significance between groups assessed by Fisher’s exact test.
(D) Area under receiver-operating characteristic (AUROC) curve values for Category I and Category II tumors representing prediction of ICB response rate across TMB threshold values, where 0.5 represents random chance and 1.0 represents perfect prediction. Rank-sum test. See Figure S6 for individual plots.
(E) Logistic regression for ability of TMB to predict response to ICB in Category I and Category II tumors, where a positive coefficient represents improved response in TMB-H tumors. TMB was taken as a continuous variable, and individual cohorts treated as random effects.

(F) Hazard ratio for overall survival following ICB treatment in TMB-H vs. TMB-L tumors, with negative log₂(Hazard Ratio) representing better outcomes in patients with TMB-H tumors. Cox proportional hazards model with cohort used as a stratification variable.

(G) Hazard ratio for overall survival following ICB treatment treating TMB as a continuous variable, with negative log₂(Hazard Ratio) representing better outcomes in patients with higher TMB tumors. Cox proportional hazards model with cohort used as a stratification variable.