A Blood-based Assay for Assessment of Tumor Mutational Burden in First-line Metastatic NSCLC Treatment: Results from the MYSTIC Study

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ABSTRACT

Purpose: Tumor mutational burden (TMB) has been shown to be predictive of survival benefit in patients with non–small cell lung cancer (NSCLC) treated with immune checkpoint inhibitors. Measuring TMB in the blood (bTMB) using circulating cell-free tumor DNA (ctDNA) offers practical advantages compared with TMB measurement in tissue (tTMB); however, there is a need for validated assays and identification of optimal cutoffs. We describe the analytic validation of a new bTMB algorithm and its clinical utility using data from the phase III MYSTIC trial.

Patients and Methods: The dataset used for the clinical validation was from MYSTIC, which evaluated first-line durvalumab (anti-PD-L1 antibody) + tremelimumab (anticytotoxic T-lymphocyte-associated antigen-4 antibody) or chemotherapy for metastatic NSCLC. bTMB and tTMB were evaluated using the GuardantOMNI and FoundationOne CDx assays, respectively. A Cox proportional hazards model and minimal P value cross-validation approach were used to identify the optimal bTMB cutoff.

Results: In MYSTIC, somatic mutations could be detected in ctDNA extracted from plasma samples in a majority of patients, allowing subsequent calculation of bTMB. The success rate for obtaining valid TMB scores was higher for bTMB (809/1,001; 81%) than for tTMB (460/735; 63%). Minimal P value cross-validation analysis confirmed the selection of bTMB ≥20 mutations per megabase (mut/Mb) as the optimal cutoff for clinical benefit with durvalumab + tremelimumab.

Conclusions: Our study demonstrates the feasibility, accuracy, and reproducibility of the GuardantOMNI ctDNA platform for quantifying bTMB from plasma samples. Using the new bTMB algorithm and an optimal bTMB cutoff of ≥20 mut/Mb, high bTMB was predictive of clinical benefit with durvalumab + tremelimumab versus chemotherapy.

Introduction

Immune checkpoint inhibitors targeting programmed cell death-1 or its ligand (PD-L1) as monotherapy or in combination with anticytotoxic T-lymphocyte–associated antigen-4 therapy have shown remarkable clinical outcomes in a wide variety of cancer types compared with standard-of-care therapy (1–8); however, a significant proportion of patients with cancer do not respond to these therapies. To mediate antitumor responses in the presence of immune checkpoint inhibitors, activated tumor-infiltrating T lymphocytes need to recognize the tumor antigens/neoantigens presented at the tumor cell surface (9, 10). Among many factors that may contribute to the response, tumor mutational burden (TMB) derived from the total somatic mutation count, a surrogate for tumor neoantigen load, has been shown to correlate with the efficacy of immune checkpoint inhibitors in clinical trials (11, 12). Recent studies have shown that higher TMB may be predictive of better clinical benefit in melanoma (13, 14), non–small cell lung cancer (NSCLC; refs. 15–17), small-cell lung cancer (18), and urothelial carcinoma (19).

In many of the previous studies, TMB was determined using whole-exome sequencing (WES) or targeted panel-based sequencing from tumor tissue samples (18, 20). However, obtaining sufficient tissue samples that represent the current tumor state is often clinically infeasible, especially for patients with advanced cancers (21–23). Moreover, tumor biopsy sampling is likely to be limited to a specific area of the tumor, which may not accurately represent the entire mutational landscape, particularly in a metastatic setting due to spatial and temporal heterogeneity (24–27). Liquid biopsies using circulating cell-free tumor DNA (ctDNA) from peripheral blood have been shown to be comprehensive and convenient for mutation profiling, have faster turnaround times, are less invasive, and incur less risk to the patient than a standard tumor biopsy (22, 28, 29). Whether TMB in blood (bTMB) can be measured using a ctDNA assay as a valid predictive biomarker to guide treatment with immunotherapy has not been extensively explored. A previous study by Gandara and colleagues showed that high bTMB was associated with improved progression-free survival (PFS) in patients with NSCLC treated with atezolizumab versus chemotherapy in the second-/third-line setting (30). In the phase II B-FIRST trial of first-line atezolizumab for NSCLC, patients with high bTMB had better survival compared with those with low bTMB (31). Similarly, in the phase III IMpower110 study, clinical benefit with atezolizumab versus chemotherapy was enhanced at higher bTMB cutoffs during first-line NSCLC treatment (32). Another smaller NSCLC study (n = 66) found that high bTMB was associated with PFS benefit during first-line pembrolizumab-based therapy (33).
Translational Relevance

Tumor mutational burden (TMB) is an emerging predictive biomarker for response to immune checkpoint blockade in non-small cell lung cancer (NSCLC). Blood TMB (bTMB) assessments using circulating cell-free tumor DNA (cfDNA) offer several practical and clinical advantages over tissue TMB (tTMB). Here, we report on the accuracy and reproducibility of the GuardantOMNI cfDNA platform for quantifying bTMB and its clinical utility, using data from the phase III MYSTIC trial (first-line durvalumab ± tremelimumab vs. chemotherapy in metastatic NSCLC). The analysis population (HR, 0.76; 97.54% confidence interval (CI), 0.56–1.02; P = 0.036); however, statistical significance was not achieved in the study for the primary endpoints of improved survival with either immunotherapy arm versus chemotherapy. Exploratory analyses in all bTMB-evaluable patients (regardless of PD-L1 expression levels) identified a bTMB threshold of >20 mutations per megabase (mut/Mb) that was predictive of optimal overall survival (OS) benefit with durvalumab plus tremelimumab versus chemotherapy in addition to improved PFS and objective response rate. These analyses also showed differentiation between durvalumab plus tremelimumab combination therapy and durvalumab monotherapy at the bTMB >20 mut/Mb cut-off point and suggested that bTMB was a more effective predictor of benefit with the combination therapy than with durvalumab monotherapy. As such, we have focused on the durvalumab plus tremelimumab combination in this analysis. Here, we report the analytic validation of the bTMB algorithm, as well as full results of its performance using the MYSTIC clinical trial data, and the correlation between bTMB and TMB measured from tissue (tTMB).

Patients and Methods

Patients and samples

The dataset used for the clinical validation in this study was from MYSTIC (NCT02453282), a phase III trial that evaluated first-line durvalumab, with or without tremelimumab, versus platinum-based chemotherapy in patients with metastatic NSCLC without known driver and resistance mutations (34). As described previously, 1,118 patients were enrolled regardless of the level of PD-L1 expression and randomized 1:1 to durvalumab, durvalumab plus tremelimumab, and chemotherapy arms (Supplementary Fig. S1). Primary endpoints, assessed in patients with PD-L1 expression on ≥25% of tumor cells, were OS for durvalumab versus chemotherapy, and OS and PFS for durvalumab plus tremelimumab versus chemotherapy. Key additional exploratory endpoints included analysis of bTMB and tTMB and their relationship with clinical outcomes (34).

The MYSTIC study was performed in accordance with the Declaration of Helsinki and the International Conference on Harmonisation Good Clinical Practice guidelines. The protocol and all modifications were approved by relevant ethics committees and regulatory authorities. All patients provided written informed consent for participation (34).

Samples used in the analytic validation of the GuardantOMNI research use only (RUO) next-generation sequencing (NGS) platform included cell-free DNA (cfDNA) extracted from cell line supernatants and healthy donor plasma. cfDNA extraction from cell line supernatants has been described previously (35). The accuracy of variant calls for single-nucleotide variants (SNVs) and insertion-deletion mutations (indels) was assessed via orthogonal WES using cell lines from the Cancer Cell Line Encyclopedia (CCLE).

TMB analysis

Tumor tissue mutation profiling was performed using the FoundationOne CDx Assay (Foundation Medicine Inc.; ref. 36) on formalin-fixed, paraffin-embedded tissue samples obtained before treatment initiation in the MYSTIC study [fresh tumor biopsies collected at screening or samples taken <3 months prior to enrollment (collected primarily for PD-L1 testing) were used]. The FoundationOne CDx assay utilizes a 315-gene panel comprising a 1.1 MB DNA footprint (coding regions only; ref. 20). Mutations were reported with at least 0.5% variant allele frequency (VAF). TMB values were subsequently determined using the proprietary algorithm described previously by Chalmers and colleagues (20).

bTMB analysis

Whole-blood samples were obtained from blood collected in K2-EDTA tubes before patients started study treatment (prior to first dose of study treatment). Plasma was separated from the cellular components, frozen, and shipped to Guardant Health for analysis using the GuardantOMNI RUO NGS assay; recommended plasma input volume for the GuardantOMNI assay is 2–4 mL. Upon receipt, samples were accessioned and cfDNA was extracted, prepared, and sequenced as described previously (35, 37, 38). The GuardantOMNI assay detects SNVs, indels, copy-number variants, fusions, microsatellite instability, and bTMB across a 2.145-Mb panel; the bioinformatics methods of variant detection have been reported previously (35, 38, 39). SNVs and indels are classified as germline or somatic using a beta-binomial algorithm that utilizes databases of germline variants, as well as local germline allele fraction information to infer the somatic origin of a variant (40). Performance of this method was tested on a set of 7,221 variants from 361 clinical plasma samples; the method improved sensitivity for somatic variants from 89% to 97%, with comparable specificity of 99.2% and 99% (Supplementary Fig. S2).

bTMB algorithm

The GuardantOMNI bTMB algorithm begins with counting all somatic nonsynonymous and synonymous SNVs and indels across 1 Mb of coding regions. Germline mutations are filtered out (39). Consistent with previous studies, gene panels that are enriched for cancer genes can overestimate TMB calculations (41, 42). As such, known driver and resistance mutations are excluded from the mutation count. Putative clonal hematopoiesis of indeterminate potential (CHIP) mutations, identified as mutations commonly annotated as
CHIP in the literature and in sequencing of healthy normal donors, are also excluded (43). Given that mutation detection in plasma samples is impacted by the amount of tumor shedding, with lower shedding resulting in progressively higher chance of undetectable mutations, as well as depth of coverage, the mutation count is algorithmically adjusted to correct for these biological and technical sample features. The adjustment is based on the highest observed VAF of somatic variants as a proxy for ctDNA fraction and on unique molecule coverage as an indicator of platform analytic sensitivity for mutation detection. This adjustment removes the mutation count dependence on the sample’s tumor shedding and molecular input, resulting in a bTMB that is largely independent of these input metrics, as reported previously (44). Similar adjustment methodologies based on tumor purity for tTMB have recently been described (45). The adjustment-corrected count, normalized by panel size, is reported as bTMB (mut/Mb).

Importantly, samples are evaluated for evidence of sufficient tumor shedding and unique molecular coverage required for accurate bTMB detection. Samples with very low tumor shedding [approximated by maximal (max)-VAF <0.3%], low molecule coverage, and/or with a large fraction of putative CHIP mutations are categorized as “TMB No-Call.” The bTMB workflow is shown in Fig. 1A, with key parameters listed in Supplementary Table S1.

Minimal P value cross-validation for optimal cutoff

On the basis of the MYSTIC results (34), bTMB may be a better biomarker for durvalumab plus tremelimumab combination therapy than for durvalumab monotherapy (where PD-L1 might be more appropriate); thus, the analyses for validation of the cutoffs were conducted only for the durvalumab plus tremelimumab versus chemotherapy arms. The MYSTIC bTMB and clinical outcome data were randomly split into two equal-sized training and validation sets. In the training set, the HRs for OS in patients with high bTMB (bTMB \( \geq \) cutoff) across a range of cutoffs for durvalumab plus tremelimumab versus chemotherapy were calculated using a Cox proportional hazards model; a minimum \( P \) value–based approach was used to select the best cutoff in the training set (46). The optimal cutoff was validated on the basis of HR values from the validation set. The above step was repeated 10,000 times. The frequency of best cutoff from the training sets and HR value distributions for each best cutoff in the validation sets were used for overall optimal cutoff assessment.

Figure 1.

bTMB analysis in the MYSTIC cohort. A, GuardantOMNI TMB workflow. B, Summary of samples analyzed for bTMB in MYSTIC (\%, percentages based on ITT population). C, VAF distribution of germline and somatic mutations in MYSTIC. D, Tumor shedding levels as assessed by max-VAF. E, Distribution of bTMB scores in the MYSTIC cohort with (inset table) proportion of patients in the MYSTIC cohort by different bTMB cutoffs. bTMB 14.5 mut/Mb corresponds to tTMB 10 mut/Mb in the MYSTIC dataset. IQR, interquartile range.
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Statistical analysis
SAS statistical software (v9.3 or above) or R (v3.5) was used for all analyses.

Data availability
The data that support the findings of this study, including the OS results and bTMB scores in individual patients by treatment arms (deidentified patient IDs), are provided in Supplementary Table S2.

Data underlying the findings described in this article may be obtained in accordance with AstraZeneca’s data sharing policy described at https://astrazenecagrouptrials.pharmacm.com/ST/Submission/Disclosure.

Restrictions included in the global MYSTIC study informed consent form prevent us from providing access to the raw sequencing reads because they include germline information; many countries have ethical and privacy concerns related to research conducted on germline sequencing information.

Results
Analytic validation
Analytic validation of the GuardantOMNI RUO platform was performed for underlying SNVs and indels, which together comprise the bTMB score. The positive percent agreement of SNVs and indels detected by GuardantOMNI was assessed against WES and CCLE publicly available data as orthogonal methods and was determined to be 840/851 (98.7%) for SNVs and 35/36 (97.2%) for indels at 30 ng of cfDNA input. The quantitative correlation of SNV VAFs as determined by GuardantOMNI versus the orthogonal WES method was high (Pearson $r = 0.995$; Supplementary Fig. S3A and S3B).

Specificity was assessed by processing a pool of cfDNA from 12 healthy donors in 24 technical replicates. Undiluted samples from these donors were also processed separately; any variants detected in the undiluted samples were removed from the analysis of the pool, as true positives. No reportable false positive SNVs were observed, for a false positive rate per megabase of <0.2%, or <1/24 per sample. One reportable false positive indel was observed, for a false positive rate per megabase of 4.2% (1/24 per sample).

Reproducibility of the quantitative TMB score was assessed in 32 replicates from 11 randomly selected clinical samples, across a clinically relevant range of bTMB scores for NSCLC. The coefficient of variation across all samples was 7.3% (Supplementary Fig. S3C). No false positive categorical TMB-high calls (based on a cutoff of 20 mut/Mb in the MYSTIC study) were observed across 52 healthy donor samples processed on GuardantOMNI.

MYSTIC samples for bTMB analysis
In MYSTIC, the median sample plasma volume available for retrospective analysis was 1.7 mL (range, 0.2–3.5 mL), yielding a median 17.2 ng of extracted cfDNA (range, 0.32–1,000 ng) in 1,001 patients with available samples. A maximum of 30 ng of extracted cfDNA (22% of samples were processed with 30 ng input) was prepared and sequenced using the GuardantOMNI assay. The distribution of plasma volume and the corresponding assay success/failure cases are shown in Supplementary Fig. 54.

Mutation and bTMB data in the MYSTIC cohort
Among 1,118 patients in the MYSTIC intention-to-treat (ITT) population (described previously; ref. 34), 1,001 had plasma samples available for cfDNA analysis (Fig. 1B). Of these, 58 samples failed quality control (QC) metrics, largely related to low sample input volumes; the success rate was 97% in 603 patient samples with plasma volume ≥2 mL, the minimum input recommended by Guardant Health. A total of 943 samples were sequenced and analyzed by the bTMB algorithm (described in the Patients and Methods section); the analysis workflow is represented in Fig. 1A, with key parameters listed in Supplementary Table S1. Of the sequenced samples, 809 had valid bTMB scores and comprised the bTMB-eligible population in MYSTIC (Fig. 1B).

Across the 943 samples that passed sequencing QC, a total of 27,610 SNVs and indels of noncommon (i.e., not present in the ExAC database with an allele frequency >0.1%) variants status were detected on the GuardantOMNI panel. Of these, 11,490 were identified as private germline mutations, 2,251 were filtered as putative CHIP mutations, and 13,869 were of somatic origin (Fig. 1C). Although there is a strong association between VAF and germline/somatic status, VAF alone was insufficient to classify germline/somatic origin; a method based on nearby common germline variants improved classification (see the Patients and Methods section). Figure 1C shows the VAF distribution of somatic and germline variants in the MYSTIC study.

In MYSTIC, tumor shedding, as assessed by max-VAF, ranged from 0% (no somatic mutations) to 90% (very high tumor fraction), with a median of 4% in 943 samples that passed sequencing QC (Fig. 1D). A total of 11.2% (91/809) of the samples had a max-VAF below 1%, a tumor shedding cutoff used in other bTMB algorithms, but still had evaluable bTMB scores by the assay used in this study. The amount of cfDNA extracted from input plasma also ranged widely, from 0.32 to >1,000 ng, resulting in a median molecule coverage of below 500 to above 5,000. The raw somatic mutation count was lower in samples with low max-VAF or low coverage (Fig. 2A and B). After applying the bTMB algorithm’s adjustment for shedding and coverage, the majority of this association was removed and the reported bTMB scores had minimal association with max-VAF or coverage (Fig. 2C and D).

Samples were assessed to determine the effect of tumor shedding on bTMB evaluable (as noted previously, 943 samples were sequenced, of which 809 had valid bTMB scores). Of the 134 TMB No-Call samples, 41 were low shedders (<0.3% max-VAF), 39 had low molecule coverage, 51 had predominantly CHIP genes among the somatic mutations, and the remaining three samples had possible contamination (Fig. 1B).

As low-shedding samples were nonevaluable for bTMB, to determine whether this introduces any bias in the resultant TMB data obtained, we used tumor tissue mutation profiling to further investigate TMB values in low-shedding samples. There was no apparent difference in the iTMB values when comparing low-shedding samples with shedding samples (Supplementary Fig. S5), although the sample size for the low shedders was small ($n = 12$). This is also consistent with our data from a small set of procured NSCLC samples showing no difference in iTMB values between low-shedding and high-shedding samples (data not shown).

The bTMB score distribution for the bTMB-eligible population in MYSTIC ($n = 809$) was long tailed with a median (interquartile range) of 13.4 (8.5–20.1) mut/Mb (Fig. 1E). The percentages of bTMB-high patients at various bTMB cutoffs, some of which have been used in previous studies that evaluated the association of bTMB with clinical outcomes (30–32), are also shown (inset table in Fig. 1E). The putative bTMB cutoffs used in some of the previous studies were based on their equivalence with iTMB cutoffs initially identified and validated in the CheckMate studies (17, 30–32, 47). Of note, the bTMB-high population based on the ≥20 mut/Mb cutoff (which was demonstrated previously to be optimal in predicting clinical benefit with durvalumab
plus tremelimumab vs. chemotherapy; ref. 34) comprises 26.1% of the bTMB-evaluable population in MYSTIC. In MYSTIC, the bTMB threshold of \( \geq 20 \text{ mut/Mb} \) was identified as optimal on the basis of improvements in OS and PFS for the combination of durvalumab plus tremelimumab versus chemotherapy and the optimal balance observed between survival benefit and the proportion of patients who derived the benefit; the cutoff selection was not based on equivalence to tTMB.

Correlation of mutations and TMB results based on the bTMB and tTMB assays

As described previously, the bTMB-evaluable population in MYSTIC comprised 809 patients (72.4% of the MYSTIC ITT population). In total, 735 (65.7% ITT) tumor tissue samples were available for testing; of these, 460 (41.1% ITT) had valid tTMB results. TMB data available using the bTMB and tTMB assays in the MYSTIC trial were balanced across the three treatment arms in the study (Fig. 3A). The success rate for achieving valid TMB scores was 18% higher for the bTMB assay (success rate 809/1,001; 81%) than for the tTMB assay (success rate 460/735; 63%). Of the 735 patients with available tissue samples, 275 did not produce a usable tTMB result due to a variety of reasons, including insufficient tumor tissue and/or tumor cells (66.2%), insufficient DNA extracted (12.3%), or failure to yield a good quality NGS library from DNA (21.5%).

Using matched tumor and plasma clinical samples from the MYSTIC trial (\( n = 352 \), 31.5% ITT), bTMB and tTMB scores were compared to assess concordance; results showed a positive correlation between bTMB and tTMB values (Spearman rho = 0.6 (95% CI, 0.51–0.67); Fig. 3B; ref. 34).

To investigate whether intratumoral heterogeneity contributed to the differences in bTMB and tTMB values, the correlation between mutations detected by blood- and tissue-sequencing assays across a common 0.61 Mb interface was evaluated in 431 patients (38.6% ITT) with both blood and tissue mutation data available. Concordance was determined using the Jaccard index, defined as the fraction of shared mutations in both blood- and tissue-sequencing assays among the entire mutation set from both tissue and blood samples. The overlapping and mutually exclusive variants, identified using the two assays with a \( \geq 0.5\% \) VAF cutoff, are depicted in the Venn diagram (Fig. 3C). Approximately 50% concordance was observed for mutations identified in tissue and blood samples, which is comparable with data reported previously (30). The proportion of unique mutations was 29% and 21% for the tissue and blood assays, respectively.

Assessment of robust bTMB cutoff associated with clinical benefit

Using the GuardantOMNI bTMB assay and algorithm described here, we have demonstrated previously that, in the MYSTIC study, high bTMB was predictive of OS and PFS benefit when comparing durvalumab plus tremelimumab with chemotherapy (34). As MYSTIC was the only dataset available for the evaluation of the optimal bTMB cutoff, without an additional independent validation set, we tested the robustness of various bTMB cutoffs using a minimal P value cross-validation analysis (as described in the Patients and Methods section). From 10,000-time repeats of random resampling of a training set from the MYSTIC bTMB data, bTMB cutoffs of 11, 12, 17, and 20 mut/Mb were most frequently selected, suggesting that these were potentially robust cutoffs which could predict OS benefit for durvalumab plus tremelimumab versus chemotherapy (Fig. 4A). Of these cutoffs, bTMB \( \geq 20 \text{ mut/Mb} \) showed the most favorable median OS HR for durvalumab plus tremelimumab versus chemotherapy in the validation set (Fig. 4B), confirming the previously reported selection of this cutoff for optimal clinical benefit with durvalumab plus tremelimumab (34).
Enrichment of mutations in genes within epithelial–mesenchymal transition and DNA damage response pathways in patients with high levels of TMB

Gene mutations associated with high TMB were identified using an association test, and Ingenuity Pathway Analysis (QIAGEN) was used to test for the enrichment of these genes in specific pathways. Using the bTMB and tTMB data, similar results were obtained with regard to the pathways [such as epithelial–mesenchymal transition (EMT), BRCA1, and DNA damage response (DDR) signaling pathways] that were identified as mutation-enriched pathways in the TMB-high populations (Fig. 5). There was an insufficient number of mutated genes associated with the low TMB groups (bTMB <20 mut/Mb or tTMB <10 mut/Mb) to perform pathway analysis.

Discussion

In this study, we have shown that somatic mutations could be detected in ctDNA extracted from plasma samples in the majority of patients with NSCLC enrolled in the MYSTIC trial, allowing subsequent calculation of bTMB. We further demonstrated that the optimal cutoff of bTMB ≥20 mut/Mb identified using a Cox proportional hazards model and minimal P value cross-validation approach was...
predictive of OS and PFS benefit from durvalumab plus tremelimumab versus chemotherapy in MYSTIC (34). The success rate for obtaining valid bTMB values from the plasma samples was substantially higher (81%) than that for obtaining valid tTMB values (63%) in MYSTIC. Higher assay failure rates with tTMB could be due to a variety of reasons, including insufficient tissue and/or tumor cells, insufficient DNA extracted, or a poor-quality NGS library. The easier availability of blood versus tissue samples seen in general in NSCLC trials and the higher assay success rates with blood samples compared with tissue samples that we observed in MYSTIC are important, as the size of the biomarker-evaluable population will directly determine the feasibility of using the relevant biomarker to define the patient population for a key clinical endpoint in a clinical trial.

Insufficient tumor DNA shedding into blood was observed for 4% (41/943) of plasma samples that passed sequencing QC; these comprised 31% (41/134) of bTMB nonevaluable plasma samples in MYSTIC. The samples with insufficient tumor shedding could not reliably be used to measure bTMB; such samples were, therefore, deemed nonevaluable in the GuardantOMNI bTMB algorithm. However, the rates of nonevaluable samples with the bTMB assay were lower than that with the tTMB assay (19% vs. 37% of available samples, respectively). In addition, approximately 11% of patients had <1%
Enrichment of mutations in genes within EMT and DDR pathways in patients with high levels of TMB

Figure 5.
Enrichment of mutations in genes within EMT and DDR pathways in patients with high levels of TMB (bTMB ≥20 mut/Mb or tTMB ≥10 mut/Mb).

max-VAF (a tumor shedding cutoff used in other bTMB algorithms), but were evaluable for bTMB using the current algorithm.

The degree of positive correlation between bTMB and tTMB data [Spearman rho = 0.6 (95% CI, 0.51–0.67)] observed in MYSTIC was similar to that shown in a previous study in patients with advanced NSCLC (Spearman rho = 0.64; ref. 30), in which the same DNA-targeted panel and very similar TMB algorithms were used for both the bTMB and tTMB assays. Several factors can affect the degree of correlation between bTMB and tTMB, including differences in DNA-targeted panels, assay technologies, and algorithms for TMB assessment, as well as known intratumoral heterogeneity in advanced NSCLC (48, 49). In our study, we found a concordance (defined by the Jaccard index) of approximately 50% between the mutations identified by blood- and tissue-sequencing assays, which is comparable with the data reported previously in a smaller cohort of patients (n = 22; ref. 30). One factor contributing to the approximate 50% difference in variants observed between blood and tissue samples could be the substantial intratumoral heterogeneity in the NSCLC cohorts studied here and previously (30). Additional factors may also include differences in assay panels, technologies, and TMB algorithms used between the two platforms.

As an emerging predictive biomarker of clinical benefit with immunotherapy that may have certain advantages over tTMB, bTMB has been tested in multiple studies using NGS tumor-profiling panels (30–33). In a previous study using bTMB data from the OAK/POPLAR trials, high bTMB was shown to be predictive of PFS benefit with atezolizumab treatment (30). However, these analyses did not show a similar predictive effect of high bTMB on OS benefit. More recently, the phase II B-FIRST trial of atezolizumab for first-line treatment of NSCLC demonstrated patients with high bTMB had numerical benefit for both PFS and OS compared with those with low bTMB; a similar trend was also seen with first-line pembrolizumab-based therapy where patients with higher bTMB had better PFS (although the lack of a randomized control arm in these trials does not allow for assessment of predictivity; refs. 31, 33). Similarly, the results from the phase III IMPower110 study showed enrichment in clinical benefit with atezolizumab versus chemotherapy at higher bTMB cutoffs during first-line NSCLC treatment (32).

One of the challenges of using TMB (whether from blood or tissue) as a biomarker is determining the optimal cutoff, as TMB is a continuous numeric variable rather than a discrete one. Several bTMB cutoffs (10, 12, 14.5, and 16 mut/Mb) have been shown previously to be clinically meaningful (30–32). A previous study validating a bTMB assay (30) used data from two trials to determine the optimal bTMB cutoff; POPLAR samples were used as a training set and OAK samples as a validation set. Our analysis consisted of a single NSCLC dataset from MYSTIC and thus the data were split randomly into two equal parts for training and validation to determine a robust candidate cutoff that is predictive of optimal OS benefit in NSCLC. Use of this cross-validation method in this study confirmed the previously reported identification of bTMB ≥20 mut/Mb as the cutoff that rendered optimal OS improvement for the combination of durvalumab plus tremelimumab versus chemotherapy and differentiation versus durvalumab monotherapy (34). However, it is important to note that the MYSTIC trial was not designed to test bTMB or tTMB; the samples were not collected with the intent of performing such analyses. Hence, there were limitations in the availability of tissue and blood samples to perform this exploratory analysis. Further evaluation of success rates in tTMB and bTMB analysis, and validation of the bTMB algorithm, as well as confirmation of the optimal cutoff in additional randomized controlled studies are required.

The pathway analysis in this study showed enrichment of mutations in the EMT, BRCA1, and DDR signaling pathways in the TMB-high populations (assessed using both bTMB and tTMB). The BRCA1 pathway in DDR signaling is known to have mutations in multiple genes that can correlate with higher TMB, such as BRCA1/2 (50). ARID1A, one of the most frequently mutated genes in cancer, was also shown here to be one of the DDR genes that correlated with high TMB. It is now known to be an important gene that regulates chromatin remodeling by associating with MSH2 for mismatch repair (51). The enrichment of mutations in genes associated with EMT-related
pathways indicates that loss of epithelial differentiation may represent one of the common mechanisms exploited by tumor cells to progress (52, 53).

In conclusion, our study shows the feasibility, accuracy, and reproducibility of a novel algorithm for quantifying bTMB from plasma samples. The bTMB analysis in MYSTIC showed a higher assay success rate than tTMB and is less invasive. Using the new bTMB algorithm and an optimal bTMB cutoff of ≥20 mut/Mb, we observed that high bTMB was predictive of both PFS and OS (34). Such benefit reported in MYSTIC may render bTMB a promising predictive biomarker for checkpoint blockade–based immunotherapy.

**Authors’ Disclosures**

H. Si reports employment at AstraZeneca during the conduct of the study. M. Kuziura reports personal fees from AstraZeneca during the conduct of the study. K.J. Quinn reports a patent for PCT/US2019/042882 pending to Guardant Health Inc. E. Helman reports a patent for PCT/US2019/042882 pending. J. Ye and F. Liu report employment at AstraZeneca during the conduct of the study. U. Scheuring reports personal fees from AstraZeneca during the conduct of the study. S. Peters reports personal fees from AbbVie, Amgen, AstraZeneca, Bayer, Biscartis, Biowint, Blueprint Medicines, Boehringer Ingelheim, Bristol Myers Squibb, Clovis, Daichi Sankyo, Debiopharm, Eli Lilly, F. Hoffmann-La Roche, Foundation Medicine, Illumina, Janssen, Merck Sharp and Dohme, Merck Serono, Merrimack, Mirati, Novartis, Pharmacia, Pfizer, Regeneron, Sanofi, Seattle Genetics, Takeda, and Vaccibody, and grants from Amgen, AstraZeneca, Biodexis, Boehringer Ingelheim, Bristol Myers Squibb, Clovis, F. Hoffmann-La Roche, Illumina, Merck Sharp and Dohme, Merck Serono, Novartis, and Pfizer outside the submitted work. N.A. Rizvi reports personal fees from AstraZeneca during the conduct of the study, personal fees from AbbVie, Apricity, BMS, Boehringer, Calithera, Dracen, Editas, EMD Sorono, G1 Therapeutics, Genentech, Gilead, GlososmithKline, Illumina, Janssen, Lilly, Merck, Neogeneomics, Novartis, Pfizer, Regeneron, and Takeda, personal fees and other from Bellcicum, Biome, Immunotherapeutics, Arcus, and Grifstone outside the submitted work, and also has a patent for PCT/US2015/060218 pending and with royalties paid from PGDX. P.Z. Brohawn reports personal fees from AstraZeneca outside the submitted work, and also has a patent for blood-based TMB pending. K. Ranade reports employment at AstraZeneca during the conduct of the study. K.C. Banks reports employment at and stock ownership in AstraZeneca during the conduct of the study, and also has a patent for AstraZeneca pending. K.C. Banks reports employment at Guardant Health during the conduct of the study. V.K. Chand reports employment at AstraZeneca during the conduct of the study, and stock ownership in AstraZeneca and Bristol Myers Squibb outside the submitted work. R. Raja reports employment at and stock ownership in AstraZeneca outside the submitted work, and also has a patent for tumor mutation burden pending.

**Authors’ Contributions**

H. Si: Writing-review and editing, collected, analyzed, and interpreted data, involved in writing or critically reviewing the report, and approved the final version. M. Kuziura: Writing-review and editing, collected, analyzed, and interpreted data, involved in writing or critically reviewing the report, and approved the final version. K.J. Quinn: Writing-review and editing, collected, analyzed, and interpreted data, involved in writing or critically reviewing the report, and approved the final version. E. Helman: Writing-review and editing, collected, analyzed, and interpreted data, involved in writing or critically reviewing the report, and approved the final version. J. Ye: Writing-review and editing, analyzed data, involved in writing or critically reviewing the report, and approved the final version. F. Liu: Writing-review and editing, analyzed data, involved in writing or critically reviewing the report, and approved the final version. S. Peters: Writing-review and editing, involved in writing or critically reviewing the report, and approved the final version. N.A. Rizvi: Writing-review and editing, involved in writing or critically reviewing the report, and approved the final version. P.Z. Brohawn: Writing-review and editing, involved in writing or critically reviewing the report, and approved the final version. K.C. Banks: Writing-review and editing, involved in writing or critically reviewing the report, and approved the final version. B.W. Higgs: Writing-review and editing, involved in writing or critically reviewing the report, and approved the final version. K. Ranade: Writing-review and editing, involved in writing or critically reviewing the report, and approved the final version. R. Raja: Writing-review and editing, involved in writing or critically reviewing the report, and approved the final version.

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**References**


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