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The Challenges of Tumor Mutational Burden as an Immunotherapy Biomarker

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SUMMARY

Tumor mutational burden (TMB) reflects cancer mutation quantity. Mutations are processed to neo-antigens and presented by major histocompatibility complex (MHC) proteins to T-cells. To evade immune eradication, cancers exploit checkpoints that dampen T-cell reactivity. Immune checkpoint inhibitors (ICIs) have transformed cancer treatment by enabling T-cell reactivation; however, response biomarkers are required, as most patients do not benefit. Higher TMB results in more neo-antigens, increasing chances for T-cell recognition, and clinically correlates with better ICI outcomes. Nevertheless, TMB is an imperfect response biomarker. A composite predictor that also includes critical variables, such as MHC and T-cell receptor repertoire, is needed.

Introduction

Immune checkpoint inhibitors (ICI) are profoundly altering the therapeutic landscape for many cancers. An inhibitor of the T lymphocyte-associate antigen 4 (CTLA-4) and six inhibitors of the programmed cell death protein pathway (PD1/PD-L1) have been granted Food and Drug Administration (FDA) regulatory approval in multiple malignancies. (Bellmunt et al., 2017; Brahmer et al., 2015; Hodi et al., 2010; Jardim et al., 2018; Motzer et al., 2015; Robert et al., 2015; Seiwert et al., 2016) Even so, overall response rates (RRs) with these agents as monotherapy is low (~15%-20%), but some individuals can attain

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durable complete remissions. (Borghaei et al., 2015; Ikeda et al., 2016; Shitara et al., 2018; Siefker-Radtke et al., 2018) Unfortunately, ICIs can also cause a number of unique immune-related toxicities (Patel and Kurzrock, 2015; Postow and Hellmann, 2018) as well as accelerated progression, termed hyperprogression, in a subset of treated individuals. (Champiat et al., 2017; Kanjanapan et al., 2019; Kato et al., 2017)

The variability of response to ICIs highlights the need for identifying predictive biomarkers. PD-L1 expression measured by immunohistochemistry (IHC) is one of the most intuitive predictive biomarkers and, in fact, can enrich the selection of candidates who may respond to ICIs. However, despite its frequent adoption in clinical practice, PD-L1 expression is associated with multiple limitations, including technical ones.(Patel and Kurzrock, 2015; Topalian et al., 2016). Another biomarker that has recently garnered significant attention is tumor mutational burden (TMB), which is a measure of the number of mutations in a cancer; the more mutations, the more neoantigens and the higher the chances that one or more of those self neo-antigens will be immunogenic and trigger a T-cell response. Since TMB was first recognized as a potential biomarker for ICIs in melanoma (Snyder et al., 2014), many studies have reported a connection between higher TMB and ICI efficacy, suggesting that TMB could be a good predictive biomarker (Tables 1 and 2).(Buttner et al., 2019; Chalmers et al., 2017; Goodman et al., 2017; Samstein et al., 2019; Turajlic et al., 2017) However, PD-L1 expression and TMB are not significantly correlated within most cancer subtypes. (Cristescu et al., 2018; Patel and Kurzrock, 2015; Yarchoan et al., 2019) Major challenges for TMB utility are the observations that TMB may not always correlate with ICI responsiveness (Paz-Ares et al., 2019) and the proper integration of TMB assessment with other ICI response/resistance biomarkers.

Definition and Experimental Determination of TMB

The conceptual definition of TMB is total number of mutations present in a tumor specimen. The actual definition of the type of genetic alterations considered for TMB has varied according to different methodologies (Figure 1). Characterization of TMB was initially performed using whole exome sequencing (WES), which by its nature considers genetic alterations restricted to exomes (coding regions). TMB calculation by WES included non-synonymous mutations in coding regions and excluded germline alterations by subtracting matched normal samples. Due to the technical complexity and high cost of WES, comprehensive gene panels using next generation sequencing (NGS) have also been used in the clinic as a substitute for WES. (Campesato et al., 2015; Johnson et al., 2016; Roszik et al., 2016).

A comprehensive genomic profiling assay of 324 genes (corresponding to 1.1 Mb of coding genome) developed by Foundation Medicine (Cambridge, MA, USA) was validated as an accurate assessment of TMB estimated by WES (Table 1).(Chalmers et al., 2017) This assay was recently approved by the FDA as a companion diagnostic for pembrolizumab in patients with TMB ≥10 mutations/Mb.(FDA, 2020) The FoundationOne assay also includes in its TMB estimation synonymous mutations and short indels in intronic regions, which are excluded from WES. Indels usually create novel DNA open reading frames, leading to an entirely new stretch of peptides that perhaps have a higher chance of encoding immunogenic

neo-antigens. Indeed, prior clinical observations suggested better responsiveness to ICIs in patients with higher burden of indels, especially in renal cell carcinoma (Turajlic et al., 2017), though this may not hold true for other solid tumors.(Wood et al., 2020). Another USA FDA-approved NGS assay is the MSK-IMPACT developed by Memorial Sloan Kettering. This test currently identifies somatic exonic mutations in 468 cancer-related genes (approximately 1.2 Mb).(Samstein et al., 2019)

A linear relationship between TMB and ICI responsiveness has been described. (Goodman et al., 2017) However, there is currently no consensus in the definition of TMB cut-offs for patient stratification. FoundationOne calls high TMB (TMB-H) when ≥20 mutations/Mb are detected; TMB-Intermediate, 6-19 mutations/Mb; and TMB-Low, ≤5 mutations/Mb . (Chalmers et al., 2017; Goodman et al., 2017) Nonetheless, the recent tissue-agnostic FDA approval of pembrolizumab defined elevated TMB as being ≥10 mutations/Mb. Since the absolute TMB numbers are quite variable between histologies, another potential approach would be to consider the top 20% of TMB for each histology (Samstein et al., 2019).

TMB assay harmonization

There is a need for standardization of tissue TMB calculation and reporting to ensure reproducibility.(Buttner et al., 2019; Fancello et al., 2019) Recent data generated in cohorts of patients with lung cancer suggested that harmonization of targeted panels is possible through normal transformation followed by standardization to *z* scores, resulting in a linear relationship.(Vokes et al., 2019) An ongoing effort to reconcile TMB methodologies is being conducted by Friends of Cancer Research (https://www.focr.org/events/tmb-harmonization-working-group-meeting).

TMB estimation using blood-based tests

Circulating tumor DNA (ctDNA) (blood biopsy) has also been used for TMB estimation. (Davis et al., 2017; Gandara et al., 2018; Khagi et al., 2017; Muller et al., 2017) The convenience of noninvasive sample collection and the possibility of repeated sampling during therapy are some of the potential advantages for this technique. Early paired comparisons for TMB estimation between tissue and ctDNA showed a lack of concordance between the techniques in some studies (Davis et al., 2017). More recently, sophisticated techniques and algorithms to evaluate blood TMB have yielded consistent promising results, and multiple studies also demonstrate the correlation between blood TMB-H and immunotherapy response (Table 2).(Gandara et al., 2018; Khagi et al., 2017; Kim et al., 2018; Peters et al., 2019; Wang et al., 2019) The use of blood-based TMB assessment may be especially important since a subgroup of patients (~15% in our experience) may not have tissue for TMB assessment, either because the tissue is unavailable or the tissue quality is not adequate.

TMB and Tumor Immune Response

Immune evasion is a hallmark of cancer. T-cells normally recognize neo-antigens produced as a result of mutations and presented by major histocompatibility complex (MHC) proteins on the surface of cancer cells, and target those cells for destruction. In order to survive, the tumor hijacks proteins that normally serve as checkpoints which attenuate immune response

against healthy tissues. By blocking immune checkpoint proteins, such as PD-1, PD-L1 and CTLA-4, with ICIs, the immune system can be reawakened. However, once reactivated, the T-cells must still be able to differentiate tumor from normal cells. Recognition of cancer cells is facilitated if there are immunogenic neo-antigens presented on their surface. Since neo-antigens are produced as a result of mutations, the greater the number of mutations (i.e., the higher the TMB), the greater the chance that some of the neo-antigens presented by MHC proteins will be immunogenic, and hence enable T-cell recognition and cancer cell eradication. (Chabanon et al., 2016; Chalmers et al., 2017; Rooney et al., 2015), On this basis, it is not surprising that TMB-H correlates with better outcome after ICI therapy (Tables 1 and 2). Nonetheless, the variable attrition rates between genomic events captured by TMB and the final steps of MHC presentation and immune-mediated tumor killing can explain, in part, the inter-patient variation of TMB as a predictive factor for immunotherapy responses.(Goodman et al., 2020) Ultimately, TMB has demonstrated a reasonable prediction of responses especially to ICI, but also to other immunotherapies.(Lauss et al., 2017) However, any attempt to dichotomize the predictive ability of TMB is imperfect, as T-cell recognized neo-antigens can, in theory, originate in a low mutation setting (although with lower likelihood); conversely, large numbers of mutations do not necessarily translate to immunogenic neo-antigens.

In summary, from an immune-oncology viewpoint, it is important to highlight that TMB has important limitations as a predictive biomarker, especially when used in isolation. (Schumacher et al., 2019) These limitations may explain why the response rate in patients with tumors that have TMB-H (≥20 mutations/Mb) is only ~45% (Goodman et al., 2017). First, only a small fraction of non-synonymous mutations will result in neo-antigens that are recognized by T-cells. Second, the clonality of these neo-antigens and the specific tumor molecular signatures contribute to the ability to generate a unique and effective anti-tumor response.(Matsushita et al., 2012) Third, factors related to the host immunological microenvironment can impact T-cell mediated tumor killing: ability of T-cells to traffic to the tumor site, the balance between activating and suppressive cytokines, the type of checkpoint exploited by the tumor, and the regulation of metabolic pathways that altogether will lead to an inflamed (hot) tumor microenvironment. Host MHC and T-cell receptor (TCR) landscape also play an important part in immune responsiveness. (Blank et al., 2016) Therefore, while TMB-H correlates with better outcomes after ICI administration, the complexity of the immune response means that TMB must be considered along with multiple other factors in order to optimize prediction of ICI outcome.

Genomic Interactions Impacting TMB as a Biomarker

TMB reflects the mutagenic processes induced by environmental and intracellular factors (Figure 2). The association between TMB and mutational signatures, including those related to carcinogen exposure or endogenous mutagenic processes, such as deficient mismatch repair (dMMR) (associated with MSI-H and TMB-H), are currently the focus of many ongoing research analyses. MHC, TCR repertoire, and specific genomic alterations also play an important role in determining ICI outcome.

Microsatellite instability-high (MSI-H) correlates with TMB-H and with ICI response

MSI-H is an important gene signature associated with TMB-H. Tumors with deficient mismatch repair (dMMR) are characterized by a higher number of uncorrected DNA defects leading to an MSI-H phenotype. (Mardis, 2019) In fact, about 83% of MSI-H tumors also present TMB-H (≥20 mutations/Mb) (Chalmers et al., 2017). Conversely, only 16% of tumors with TMB-H are also MSI-H, demonstrating that MSI-H is only one of the possible factors that lead to TMB-H.(Chalmers et al., 2017) MSI-H is described in ~4% of cancers, most frequently colorectal, gastric, and endometrial adenocarcinomas. About 15% of MSI-H cancers are due to germline mutations in MMR genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*). MSI-H may also occur because of non-hereditary epigenetic inactivation of MMR genes in addition to somatic mutations.(Bonneville et al., 2017)

In 2017, the FDA granted accelerated approval to pembrolizumab (anti-PD-1) for both adult and pediatric patients with MSI-H or dMMR solid tumors. This was the first time the agency authorized a cancer treatment based on a genomic biomarker that was histology agnostic. RRs of MSI-H solid tumors to ICIs are ~40% in the pan-cancer setting, with a subset of responses showing long-term durability.(Marcus et al., 2019) The TMB-H that characterizes most MSI-H cancers probably accounts for the immunotherapy responsiveness.

Microsatellite-stable tumors with TMB-H are responsive to checkpoint blockade

Microsatellite-stable tumors with TMB-H also benefit from ICIs.(Goodman et al., 2019b) Median progression-free survival (PFS) for microsatellite-stable/TMB-H (≥20 mutations/Mb) versus microsatellite-stable/TMB-Low/TMB-Intermediate tumors was 26.8 vs. 4.3 months (P=0.0173). Furthermore, 2,179 of 148,803 samples (1.5%) were MSI-H, while 9,762 (6.6%) were TMB-H (7,972, microsatellite-stable/TMB-H). Therefore, microsatellite-stable/TMB-H tumors are substantially more common than MSI-H cancers. (Goodman et al., 2019b) Recent results from the TAPUR study (NCT02693535) in heavily pre-treated colorectal cancer also suggest activity of pembrolizumab in microsatellite-stable/TMB-H patients. (Meiri et al., 2020) Of interest, the FDA has recently granted tissue-agnostic accelerated approval for the pembrolizumab in TMB ≥10 mutations/Mb solid tumors.(FDA, 2020) The evidence derived from a single-arm phase II trial demonstrated RRs of ~28% in patients with TMB ≥10 mutations/Mb, with 50% of the responses being durable at two years; however, survival was not increased.(Marabelle et al., 2019) Hence, from the practical perspective, TMB is an approved biomarker for selecting patients for ICI monotherapy, albeit one with an incomplete correlation with outcome.

Genomic signatures associated with exogenous mutagens impact ICI outcome

Ultraviolet (UV) light, tobacco smoking, aflatoxin B1 and benzene exposure, as well as viruses, cause mutational signatures that influence the emergence of specific genomic alterations and TMB status as well as neo-antigen immunogenicity. As a consequence, TMB is not defined by one universal mutational signature across tumors. Signatures associated with exogenous mutagens (smoking, UV light, etc) are more frequent in melanoma and lung cancer (which are considered to be tumors that frequently have TMB-H); conversely, signatures associated with DNA repair gene (MMR, POLE) defects, also associated with

TMB-H, are more predominant in endometrial, colorectal and esophagogastric cancers. (Zehir et al., 2017)

The correlation between TMB and the formation of immunogenic neo-antigens is variable and dependent on the mutational signatures (Table 3). (Alexandrov et al., 2016; Boichard et al., 2019; Kucab et al., 2019; Romualdo Barroso-Sousa, 2018). For instance, apolipoprotein B mRNA-editing cytidine-deaminase (APOBEC) signatures, characterized by C > G and C > T mutations (Alexandrov et al., 2013; Burns et al., 2013), are a result of viruses and are strongly correlated with immune activation while, for the aging process signature, the correlation is nonsignificant.(Boichard et al., 2019; Budczies et al., 2018) The APOBEC signature is also associated with the expression of PD-L1 and interferon gamma in tumor cells.(Boichard et al., 2017) APOBEC deregulation and the resulting specific mutational pattern called *kataegis* also correlates with the overall mutation burden(Boichard et al., 2017) and with DNA changes that mediate enhanced neo-peptide hydrophobicity, which is known to increase immunogenicity.(Boichard et al., 2019) Further, an APOBEC signature correlated with ICI responsiveness independently of TMB in a cohort of 99 patients. (Boichard et al., 2019) This relationship was established regardless of the presence of MSI-H. UV signature also correlated with increased neo-antigen hydrophobicity/immunogenicity, and was able to predict better response ($P = 0.0026$), PFS ($P = 0.036$), and survival ($P = 0.052$) after ICIs in patients with TMB-Low/Intermediate (<20 mutations/Mb), but not in patients with TMB-H.(Pham et al., 2020)

Major histocompatibility complex (MHC) and the TCR repertoire mediate ICI outcome

MHC diversity determines how well neo-antigens can be presented, while TCR repertoire defines neo-antigen recognition.(Chowell et al., 2018; Weber et al., 2016) As background, the function of MHC molecules is to bind peptide fragments and display them on the cell surface for recognition by the appropriate T-cells. MHC is polygenic and the heterogeneity found in its molecules plays a significant role in shaping an individual's immune reaction to malignancies. Specifically, MHC variation can greatly affect peptide binding during antigen presentation, which impacts the peptide repertoire presented on the cell surface by MHC class I or II proteins for T-cell recognition. While maintaining the ability to respond to foreign peptides, the T-cell population in an individual needs to avoid harmful activation by self-antigens. For instance, co-inhibitory surface receptors, such as PD-1 and CTLA-4 immune checkpoints, recognize surface-expressed ligands on self-tissues and act to dampen unwanted immune activation. Cancer hijacks these immune checkpoints in order to survive.

Important steps that are required before the somatic mutations that reflect TMB can in fact elicit T-cell destruction include (Chabanon et al., 2016): (i) somatic alterations need to be translated to neo-antigens that will bind to MHC at the surface of tumor cells; and (ii) altered neoantigens presented by the MHC need to be efficiently recognized by TCRs. Indeed, the likelihood of neo-antigen presentation by the MHC (Goodman et al., 2020) and of subsequent recognition by T cells (Luksza et al., 2017) characterize a predictive neo-antigen fitness model based on the host immune pharmacogenome.

MHC-I molecules are major players in shaping the mutational landscape of cancer (Mage et al., 2012; Marty et al., 2017) because of their ability to present potential neo-antigens

derived from tumor mutations. In turn, oncogenic processes and mutations negatively correlate with MHC-I presentation, supporting MHC as having a crucial role in immune-editing. Maximal heterozygosity at MHC-I loci (leading to improved host ability to present a higher number of cancer neo-antigens) is associated with longer survival in ICI-treated patients.(Chowell et al., 2018) In addition, melanoma patients receiving ICIs demonstrated that some human leukocyte antigen (HLA) subtypes (with HLA being the human version of the MHC complex), such as *HLA-B44*, are able to preferentially present certain antigens enriched in the tumor, leading to improved survival.(Chowell et al., 2018) These HLA super-types enhance the positive effects of TMB-H on survival in patients with cancer. Conversely, tumors that lose HLA expression or harbor functional disruptions mediated by alterations in β_2-microglobulin (a component of MHC class I) can be resistant to ICIs.(Rodig et al., 2018; Zaretsky et al., 2016) MHC class II may also be important in shaping response to the mutational processes in cancer development. Mutant peptides poorly bound to MHC-II are positively selected during tumorigenesis, more so than mutant peptides poorly bound to MHC-I.(Marty Pyke et al., 2018a) Hence, the MHC-II genotype complements MHC-I in selecting the mutational landscape during tumorigenesis, indirectly impacting the immunological potential of TMB (Marty Pyke et al., 2018b).

Another fundamental part of the immunologic response is the diverse processes that lead to an individual's TCR repertoire, which in turn leads to different abilities to recognize MHC-presented antigens among cancer patients. Absence of a T-cell response to an MHC-presented cancer neoantigen could be driven by the lack of TCR reactivity or removal of active TCRs from the host repertoire.(Linnemann et al., 2014) In contrast, a higher clonality of TCRs could indicate the availability of proper anti-tumor reactive T-cells. In fact, during treatment with ICIs, higher TCR clonality is associated with improved survival.(Weber et al., 2016) However, the effects of TCR clonality and diversity might vary with the type of checkpoint blockade.(Hogan et al., 2019; Hopkins et al., 2018; Reuben et al., 2019; Yusko et al., 2019)

In summary, MHC and TCR repertoire are vital determinants of the immune response. Malignancies with TMB-H are more likely to respond to ICIs due to the increased chance of having an immunogenic mutated peptide/neo-antigen presented by their MHC. The MHC-I genotype and its ability to present driver neo-antigens predicts which patients with TMB-H will respond to ICIs (Goodman et al., 2020) as does increased $CD8^+$ T-cell effector function and TCR diversity.(Hosoi et al., 2018)

Impact of specific genomic alterations on checkpoint blockade response and resistance

In addition to genomic signatures and the immune-pharmacogenome, there are a variety of genomic alterations that individually may correlate with sensitivity, resistance, and hyperprogression after immunotherapy (Table 3), sometimes independently of TMB. In the context of TMB-H, a variety of unique genomic alterations will occur, and most of them will be passenger events. But some of them can be driving the TMB-H (e.g., dMMR) or cause ICI response or resistance.

An interesting example of a genomic predictor is *PD-L1* gene amplification, which is associated with high RRs to PD-1 inhibitors in Hodgkin lymphoma (Ansell et al., 2015;

Roemer et al., 2016) as well as in solid tumors, surprisingly even in the absence of high PDL1 expression by IHC.(Goodman et al., 2018) Serpin genes correlate with autoimmunity, and mutations in some genes from this family were associated with positive responses to CTLA-4 inhibitors.(Riaz et al., 2016) Alterations in *CDK12* (Wu et al., 2018) as well as in chromatin remodeling genes *PBRM1*(Miao et al., 2018), *ARID1A* (Okamura et al., 2020) and *SMARCA4* (Tischkowitz et al., 2020) have been correlated with better ICI outcomes, though some of the data re *PBRM1* remains a matter of debate, and the mechanisms require clarification.

Genomic alterations may also predict poor outcome after ICIs. Beta-2 microglobulin (B2M) mutations disrupt MHC class I and thus diminish antigen presentation to immune active cells.(Shin et al., 2017; Zaretsky et al., 2016) Consequently, inhibition of PD1/PD-L1 interaction has little effect. *JAK*1/2 and *STK11* mutations are associated with attenuated responsiveness to immunotherapy even in a TMB-H setting, though it is unclear if *STK11* alterations are a prognostic or predictive marker.(Shin et al., 2017; Skoulidis et al., 2018) Finally, *MDM2* family amplification and *EGFR* aberrations can be associated with hyper-progression after ICIs, although the underlying mechanisms are unknown.(Kato et al., 2017).

Utility and Challenges of Using TMB as a Biomarker

Clinically, there is a substantial correlation between the TMB and objective responses to PD-1/PD-L1 inhibitors.(Chalmers et al., 2017; Goodman et al., 2017; Yarchoan et al., 2017) TMB can be responsible for 55% of the difference in response rate between cancer types (Chan et al., 2019; Yarchoan et al., 2017) and tissue TMB correlates linearly with better outcome (including RRs and PFS) to ICIs across cancers (Table 1).(Goodman et al., 2017) Series that included patients with melanoma treated with either ipilimumab (anti-CTLA-4) or a PD-1 inhibitor (Riaz et al., 2017; Snyder et al., 2014), or those with urothelial cancer treated with atezolizumab (anti-PD-L1) or nivolumab (anti-PD-1) (Balar et al., 2017; Galsky et al., 2017b; Powles et al., 2018) demonstrated improved outcome with higher TMB. In NSCLC, higher ICI RRs and longer PFS were also observed for patients with higher TMB in some studies, but not in others (Carbone et al., 2017; Hellmann et al., 2018b; Hellmann et al., 2018c; Rizvi et al., 2018; Rizvi et al., 2015), though in the latter, patients in the arm receiving ICIs also received chemotherapy, which could have confounded the results.(Paz-Ares et al., 2019). Importantly, in the studies with lung cancer, higher TMB has generally failed to show an overall survival advantage, even when PFS is improved (Table 1).

In a series of 151 patients with a variety of advanced solid tumors treated with ICIs, RRs were ~5% in patients with TMB-Low (≤5 mutations/Mb); ~25%, TMB-Intermediate (6-19 mutations/Mb); ~45%, TMB-H patients (≥20 to 49 mutations/M; and ~65% in individuals with very high TMB (≥50 mutations/Mb) (Foundation Medicine stratification)(Goodman et al., 2017). Unfortunately, TMB-H does not preclude tumor progression. In fact, one of the first patients reported to present with accelerated progression during ICI therapy had a TMB-H neoplasm.(Kato et al., 2017)

Comparing squamous cell versus other histologies is also relevant.(Goodman et al., 2019a) Squamous cell tumors have higher TMB than non-squamous cell cancers, with the highest

TMB in cutaneous squamous cell tumors (with 41% demonstrating a very high TMB (≥50 mutations/Mb)). In immunotherapy-treated squamous cell cancer-bearing patients, higher TMB (≥12 mutations/Mb) correlated with significantly better outcomes; cutaneous squamous cell cancers had the highest ICI clinical benefit rate (73% versus 33% for non-cutaneous squamous cancers (p=0.008)).

The importance of TMB has been widely associated with solid tumors, but it is critical to keep in mind that the total number of mutations per coding area of genome is substantially different among tumor types (as well as among individuals within a tumor type). Hematologic cancers and astrocytoma are characterized by a low number of alterations. (Chalmers et al., 2017; Schumacher and Schreiber, 2015) However, TMB-H (≥20 mutations/Mb) may also be found in 2% of hematologic malignancies (Galanina et al., 2018a). TMB for myeloid neoplasms is generally lower than for lymphoid malignancies. Still, diffuse large B cell lymphoma (DLBCL) presents a median TMB of 10 mutations/Mb and 18.4% of cases present with TMB-H.(Chalmers et al., 2017) Although the only regulatory approval of ICI in hematological malignancies was obtained for Hodgkin lymphoma (whose hallmark is PD-L1 [*CD274*] amplification, another marker for ICI response (Goodman et al., 2018)), DLBCL is known to be responsive to ICIs (Hude et al., 2017) (RR, 41%)(Zinzani et al., 2017) It is plausible that TMB-H may be driving some of these responses in DLBCL. Although most studies suggest that higher TMB correlates with better outcomes after ICIs, some of the studies are limited because they are retrospective or no survival advantage is shown. Additional prospective studies with a variety of solid and hematological malignancies are needed to enhance our understanding of TMB as a tissue-agnostic biomarker.

Low TMB does not preclude responses to ICI

ICI can be effective, even in the low TMB settings, albeit in small percentages (~5%) of patients.(Goodman et al., 2017) For example, Kaposi sarcoma is a viral-related malignancy that is responsive to ICI. Indeed, six of nine patients (67%) who received anti PD-1 monotherapy achieved a complete or partial remission; all Kaposi lesions had TMB-Low and were PD-L1 negative.(Galanina et al., 2018b) Merkel cell carcinoma is another interesting example, for which ICI RRs in advanced disease are approximately 56%. Merkel cell carcinoma can be associated with a UV signature and TBM-H or with Merkel cell polyomavirus infection and TMB-Low. Responses were observed among both virus-positive and negative tumors.(Nghiem et al., 2016) It is plausible that those Merkel cell tumors with UV signature have a TMB-H leading to response, and those with Merkel cell polyomavirus have TMB-Low, but the viral antigens themselves are immunogenic.

Role of TMB as a prognostic marker in immunotherapy-naïve patients

It is plausible that higher TMB could act as a prognostic factor for better outcome, regardless of treatment type.(Ballman, 2015). In 5,371 patients with advanced cancers that never received ICI, there was no association between higher TMB (MSK-IMPACT assay) and improved survival (HR=1.12, P=0.11).(Samstein et al., 2019) In contrast, our series with 1,415 ICI-naïve patients with advanced cancers demonstrated that TMB-H is strongly associated with longer survival (Riviere et al., 2020). An important difference among these

series is that the latter classified TMB-H as ≥20 mutations/Mb while the MSKCC series considered TMB-H as the top 20% for each histology. The prognostic impact of TMB-H is important because it could confound predictive attribution of increased survival after ICIs.

TMB as part of a composite biomarker to predict ICI outcome

The development of accurate predictors of ICI response will require an in-depth understanding of the complexity of immune response and resistance, and integration of multiple variables into a composite biomarker that may include TMB, expression of PD-L1, PD-1 and other checkpoints and considers the cells on which they are expressed, tumor molecular signature, neoantigen immunogenicity, the ability of the host MHC to present neo-antigens produced by the cancer mutanome, specific genes associated with ICI response or resistance, immune infiltration, microbiome, and TCR repertoire.(Anagnostou et al., 2019; Boichard et al., 2019; Boichard et al., 2017; Goodman et al., 2020; Havel et al., 2019; Hwang et al., 2020; Kato et al., 2020; Kumagai et al., 2020) A conceptual model for this integration was proposed as the “cancer immunogram”, which accounts for the several players that fit into a model to predict responses to immunotherapies. (Blank et al., 2016). One of the seven-parameter classes of cancer immunogram is tumor foreignness. Although TMB has its limitations for defining quality of neoantigens, it can be considered a proxy for foreignness. The other parameters of the cancer immunogram would include biomarkers related to immune infiltration and metabolism, absence of inhibitors, checkpoint status, and tumor sensitivity to immune effectors. One of the key aspects of this model is the assumption that cancer immunograms are not static, but evolve with the disease. Hence, in a composite platform, biomarkers could be added or removed with the evolution of disease

From a practical perspective, a recent meta-analysis suggested that multiplex immunohistochemistry has better accuracy in predicting responses to ICIs, compared to PD-L1 and TMB used in isolation. Integration of a multimodality cross-platform also significantly increased accuracy in this analysis.(Lu et al., 2019) As suggested by Ott et al, TMB integration into a combined biomarker analysis should preferentially include non-overlapping biomarkers, such as inflammatory markers (PD-L1 or T-cell gene expression profile).(Ott et al., 2019) As discussed here, it would make a lot of sense to integrate TMB into models including HLA genotype and TCR clonality, but published clinical studies testing these models are currently absent. Finally, one of the additional challenges of integration of biomarkers is avoiding excessively narrowing the group of patients that are candidates for immunotherapy.

Conclusions

Multiple lines of evidence suggest that higher TMB predicts better outcome after ICI therapy, albeit imperfectly. Although the FDA has recently granted tissue-agnostic accelerated approval for the anti-PD1 pembrolizumab in TMB ≥10 mutations/Mb solid tumors (FDA, 2020) and blood tests to assess TMB are being developed, there are still many challenges for the further development of TMB as a clinical biomarker. For instance, the predictive value of TMB for combinations of immunotherapies with targeted agents or chemotherapy is not established. Furthermore, it is critical to recognize that a subset (~5%)

of patients with low TMB can respond well to ICIs and that >50% of patients with TMB-H do not respond. Reflecting immune system complexity, multiple other variables will need to be incorporated into a composite biomarker in order to make prediction of ICI outcome more accurate and fully unlock the potential benefit of immunotherapy. Moreover, broad adoption of individualized in-depth immune profiling will be necessary in order to tailor immunotherapy treatment strategies on a patient-by-patient basis. Finally, prospective randomized trials are required to establish the role of TMB and other ICI biomarkers in a variety of clinical settings.

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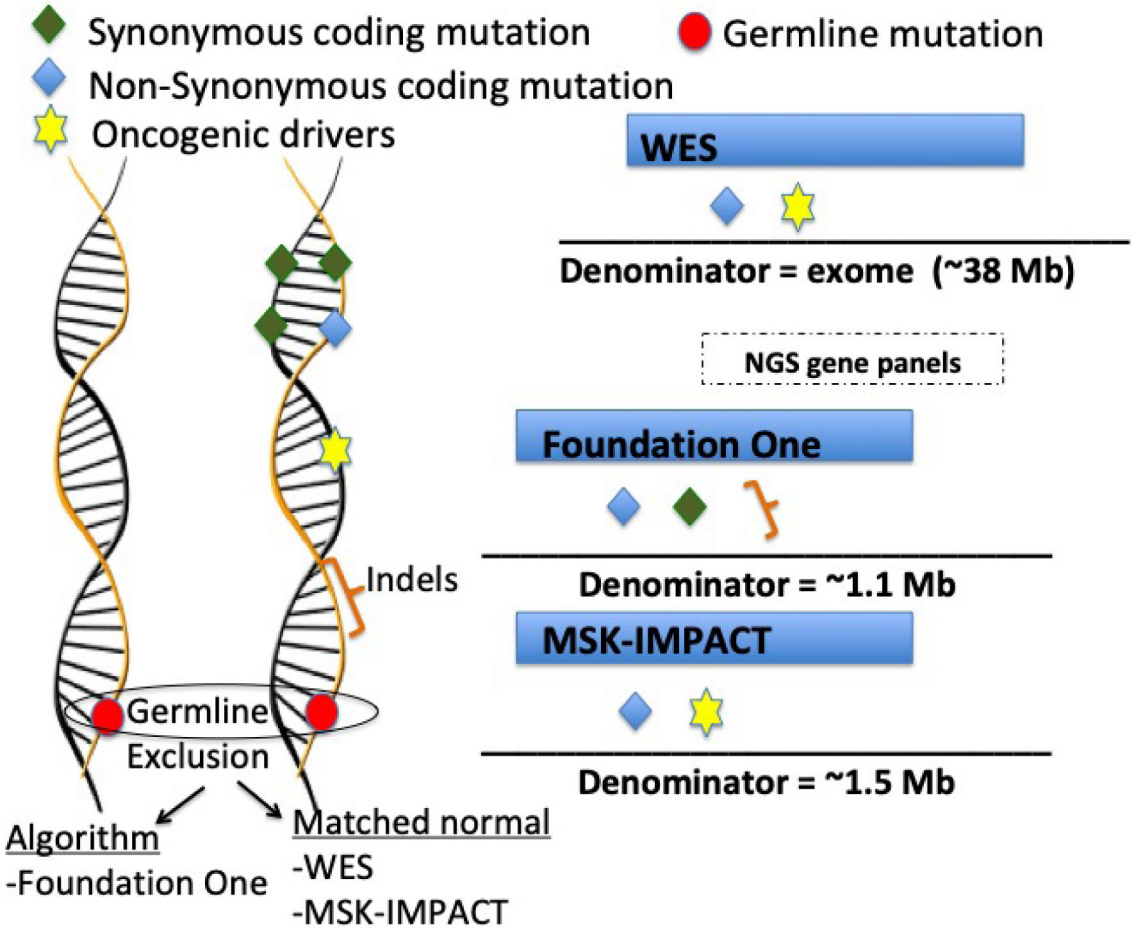

Figure 1 –. Schematic representation of the main FDA-approved assays for TMB estimation as well as whole exome sequencing calculation.

Two of the NGS gene panels are FDA-approved tests (Foundation One (Frampton et al., 2013) and MSK-Impact) (Cheng et al., 2015). Symbols represent genetic alterations that are captured as mutations, while the denominator refers to the genome region that is considered for each test. The Foundation Medicine TMB assay examines a genomic region of approximately 1.1 Mb. For TMB estimation this test includes synonymous and non-synonymous mutations and short indels, while oncogenic drivers are excluded. In addition, germline alterations are excluded based on validated bioinformatics algorithms. The MSK IMPACT TMB assay examines approximately 1.5 Mb and, similar to WES, includes non-synonymous mutations in coding regions and oncogenic drivers. Germline alterations are excluded by subtracting matched normal samples.

Examples of other commercial available assays include: Illumina TruSight 500 (~2Mb exome coverage [i.e region sequenced]), Thermo Fisher Scientific Oncomine (1.7 Mb exome coverage), Caris Molecular Intelligence (1.7 Mb exome coverage); NEO New Oncology NEOplus v2 RUO (1.1 Mb exome coverage); TruSight Tumor 170 (0.5 Mb exome coverage); Tempus Plataform (2.4 Mb exome cover)

Abbreviations: Mb = megabase; WES: Whole exome sequencing

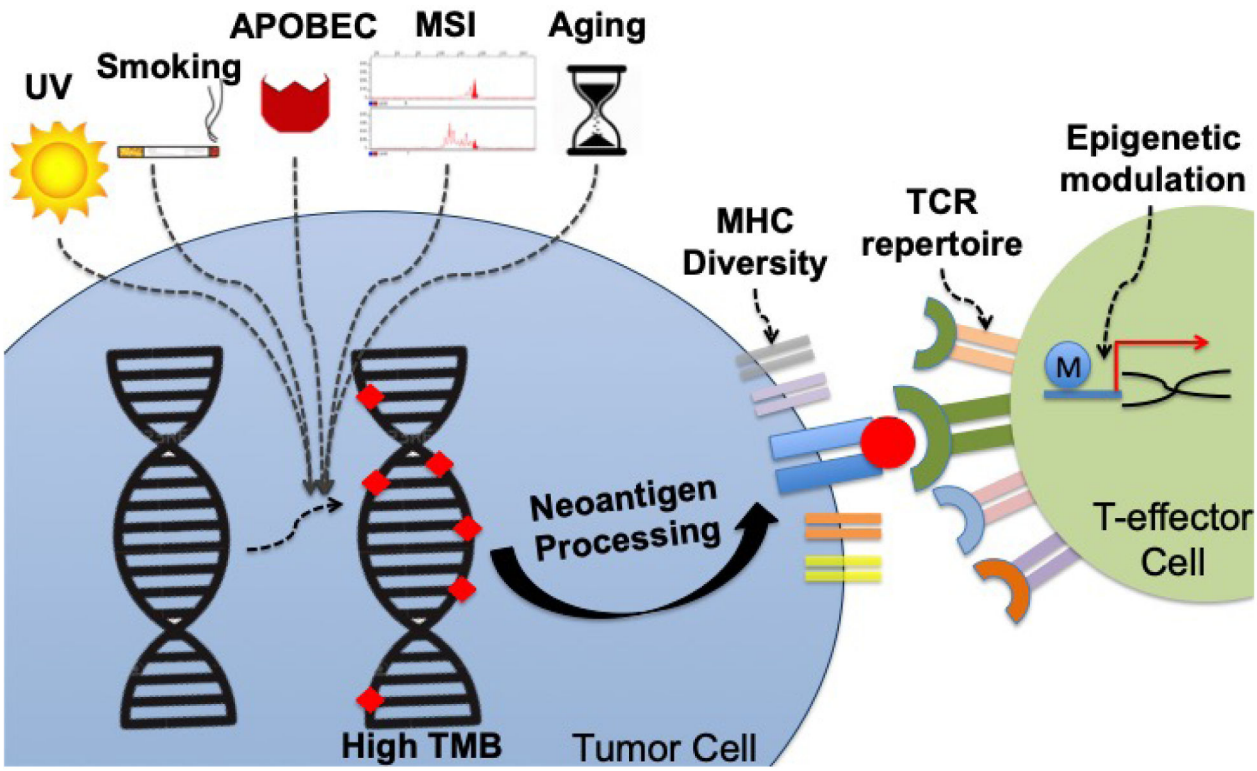

Figure 2 –. Environmental and host factors that influence TMB as a biomarker for anticancer immunotherapies.

Several different processes lead to gain of genomic alterations in tumors cells, whose number can be quantified by TMB. Environmental factors (e.g. UV) and DNA editing errors (MSI) cause patterns of mutations classified under different signatures.(Zehir et al., 2017) Each signature may influence not only the number of mutations, but also the quality and immunogenicity of the neo-antigens presented as a result of the mutanome burden. Host intrinsic characteristics also impact neo-antigen presentation and recognition. For instance, MHC diversity defines how well the neo-antigens can be presented, while TCR repertoire may define neo-antigen recognition.(Chowell et al., 2018; Weber et al., 2016) Epigenetic modulation (such as by histone modifications and DNA methylation) may also influence the host ability to generate an effective immune response.(Peng et al., 2015)

Abbreviations: MHC, major histocompatibility complex; MSI, microsatellite instability; TCR, T-cell receptor; TMB: tumor mutational burden; UV, ultraviolet light

Table 1:

Examples of tissue TMB and response to immunotherapy (checkpoint inhibitors)

Cancer type (N)	TMB and response relationship	Assay type	Treatment Strategy	Comments	References
CRPC (37 pts)	*TMB above (≥74.5 mut) vs. below (<74.5 mut) median* RR: Cohort 1 (pre-chemo): 50% vs. 9.1% (p=0.03); Cohort 2 (post-chemo): 50% vs. 0% (p=0.01) PFS (both cohorts): median 7.4 vs. 2.4 months (p<0.001)	WES	ICI+ICI	Design: exploratory analysis of phase 2 trial (CheckMate 650). (NCT02985957) Drug: ipilimumab+nivolumab Higher TMB was predictive of better RR and longer PFS	(Sharma et al., 2019)
Diverse cancers (151 pts)	*TMB high (≥20 mut/Mb) vs. low to intermediate (0-19 mut/Mb)* RR = 58% vs 20% (P=0.0001) Linear correlation between TMB and better outcome	NGS Foundation Medicine	ICI	Design: retrospective analysis of single institutional data Drugs: multiple immunotherapies (mostly ICI) TMB correlated linearly with responsiveness to checkpoint inhibitors across cancers	(Goodman et al., 2017)
Diverse cancers (30 pts)	*Higher TMB (≥8 mut/Mb) vs Intermediate/Low* Favorable Response (stable disease or response): increased probability of a favorable response with high TMB	NGS Foundation Medicine	ICI	Design: retrospective analysis of single institutional data Drugs: multiple ICIs	(Bonta et al., 2017)
Diverse cancers (119 pts)	*TMB high (≥102* mut) *vs low (<102 mut)* RR: 32.3% vs 7% (p=0.0078)	WES	ICI	Design: exploratory analysis of two prospective single arm trials (Keynote 012 and 028) (NCT01848834; NCT02054806). Drug: pembrolizumab Mutational load was only modestly correlated with interferon gene expression profile	(Cristescu et al., 2017)
Diverse Cancers (77 pts)	*Responder vs no responder* Higher TMB was significantly associated with responses (p=0.018) and longer PFS (p=0.051)	WES	ICI	Design: exploratory analysis of prospective phase Ib trial (Keynote 028) (NCT02054806). Drug: pembrolizumab Correlations between TMB with PD-L1 and T-cell inflamed gene-expression profile were low	(Ott et al., 2019)
Diverse cancers (755pts)	*TMB high (≥10 mut/Mb) vs. low (<10 mut/Mb)* RR: 28.3% vs. 6.5% (no p-value) PFS: 2.1 (95% CI, 2.1 to 3.7 months) vs 2.1 (95% CI, 2.1 to 2.3 months) (no p-value) OS: 11.1 (95% CI, 8.1 to 16.1 months) vs 13.3 (95% CI, 11.5 to 14.8 months) (no p-value)	NGS Foundation Medidine	ICI	Design: exploratory analysis of phase 2 trial (Keynote-158). (NCT02628067) Drug: pembrolizumab Led to the accelerated approval of pembrolizumab for solid tumors with TMB ≥ 10 mut/mb by the FDA	(Marabelle et al., 2019)
HNSCC (261 pts)	*TMB high (≥175 mut) vs low (<175 mut)* High TMB was associated with higher response rate and longer PFS; no association with OS was demonstrated	WES	ICI	Design: exploratory analysis of prospective trials (Keynote 012 and 055). (NCT01848834 and NCT02255097). Drug: pembrolizumab TMB was not associated with PD-L1 expression and T-cell activated gene expression profile	(Seiwert et al., 2018)
Melanoma (64 pts)	*TMB high (>100 mut per tumor) vs low (≤ 100 mut)* OS: survival advantage for high TMB (p=0.04)	WES	ICI	Design: retrospective analysis of multiple prospective clinical trials Drugs: ipilimumab or tremelimumab Patients with increased long-term benefit presented significantly higher mutational load	(Snyder et al., 2014)
Melanoma (38 pts)	*TMB top third vs bottom third* OS: improved survival (p=0.005)	WES	ICI	Design: retrospective analysis of institutional data Drugs: pembrolizumab or nivolumab High TMB predicted better survival, but not response to	(Hugo et al., 2016)

Cancer type (N)	TMB and response relationship	Assay type	Treatment Strategy	Comments	References
				anti-PD-1. BRCA2 mutations enriched in responsive patients	
Melanoma (65 pts)	*TMB high (≥23.1 mut/Mb) vs medium (3.3-23.1 mut/Mb) vs low (<3.3 mut/Mb)* RR (%): 85 vs 29 vs. 14 (p<0.001) PFS : not reached vs. 89 days vs. 86 days (p<0.001) OS: not reached vs. 300 days vs. 375 days (p<0.001)	NGS Foundation Medicine	ICI	Design: retrospective analysis of multi-institutional series Drugs: nivolumab, pembrolizumab or atezolizumab Response rate, progression-free survival, and overall survival were superior in the high, compared with intermediate and low, mutation load groups. TCR clonality did not predict response	(Johnson et al., 2016)
Melanoma (94 pts)	Tumor mutational load was associated with response to nivolumab followed by ipilimumab (p = 0.03), but not with response to ipilimumab followed by nivolumab	WES	ICI	Design: exploratory analysis of Checkmate 064 (NCT01783938) Drugs: nivolumab or ipilumumab	(Weber et al., 2016)
Melanoma (68 pts)	*TMB high (>100 mutations) vs low (≤ 100 mutations)* OS: survival advantage for high TMB (p=0.048)	WES	ICI	Design: exploratory analysis of single arm phase II trial CheckMate 018 (NCT01621490) Drug: nivolumab Mutational burden decrease after successful therapy with checkpoint blockade	(Riaz et al., 2017)
MSI-high metastatic CRC (22 pts)	*High TMB (37-41 mut) vs low TMB* RR: 100% vs. 22% PFS: not reached vs. 2 months	NGS Foundation Medicine	ICI	Design: retrospective analysis of multi institutional data Drug: PD-1/PD-L1 inhibitors TMB (as a continuous variable) showed the strongest association with an objective response (p < 0.001)	(Fakih M et al., 2019)
MSI-stable solid tumors (60 pts)	*High TMB (≥20 mut/Mb) vs. Low TMB (0-19 mut/Mb)* Benefit rate: 75% vs. 38% (OR: 3.29 [95%CI 0.91-10.29, p=0.07) PFS: 26.8 vs. 4.3 months, HR 0.42 (95% CI 0.22-0.77; p = 0.017) OS: median not reached, HR 0.4581 (95% CI 0.20-1.0; p=0.06)	NGS Foundation Medicine	ICI	Design: retrospective analysis of single institutional data Drugs: PD-1/PD-L1 inhibitors MS-Stable/TMB-High characterizes a subgroup of cancers considerably larger than the MSI-High subset, normally considered for checkpoint blockade. MSI-Stable/High TMB patients were responsive to checkpoint blockade	(Goodman et al., 2019b)
NSCLC (34 pts)	*TMB high (≥200 mut per tumor) vs low (<200 mu per tumor)* *RR: 59% vs 12% (p=0.01)* *PFS: not reached vs 3.4 months, HR 0.91 (95%CI 0.08-0.47, p<0.001))*	WES	ICI	Design: exploratory analysis of prospective phase I trial Keynote 001 (NCT01295827) Drug: pembrolizumab Higher TMB had a positive correlation with RR and PFS	(Rizvi et al., 2015)
NSCLC (312 pts)	*Nivolumab vs chemoterapy comparison* High TMB (≥243 mut): RR 47% vs 28%; PFS HR 0.62 (95% CI 0.38-1.0); OS HR 1.10 (95% 0.64-1.88) Medium and low TMB (<243 mut): RR 23% vs. 33%; PFS HR 1.82 (95% CI 1.30-2.55); OS HR 9.99 (95% CI 0.71-1.40)	WES	ICI	Design: exploratory analysis of randomized phase III trial CheckMate 026 (NCT02041533) Drug: nivolumab TMB was predictive of RR and PFS, but not OS	(Carbone et al., 2017)
NSCLC (102 pts)	*TMB high (≥13.5 mut/Mb) vs low* RR: 25% vs 20% PFS: HR 0.54 (95% CI 0.3-0.97) OS: HR 0.45 (0.17-1.16)	NGS Foundation Medicine	ICI	Design: exploratory analysis of prospective phase II trial (NCT01846416) Drug: atezolizumab Higher TMB was associated with RR and PFS in both unselected and PD-L1-selected patients	(Kowanetz et al., 2017)
NSCLC (371pts)	*TMB high (≥17.1 mut/Mb) vs low* RR: 29% vs 16% PFS: HR 0.5 (95% CI 0.38-0.67) OS: HR 0.7 (0.49-1.0)	NGS Foundation Medicine	ICI	Design: exploratory analysis of prospective phase II trial (NCT02031458) Drug: atezolizumab TMB was associated with RR, PFS and OS	(Kowanetz et al., 2017)
NSCLC (92 pts)	*Atezolizumab vs docetaxel comparison* TMB high (≥median = 15.8 mut/Mb): RR 20% vs 8%; OS= HR 0.5 (95%CI 0.15-1.67); PFS = HR 0.49 (95%CI 0.19-1.3)	NGS Foundation Medicine	ICI	Design: exploratory analysis of randomized phase II trial (NCT01903993) Drug: atezolizumab	(Kowanetz et al., 2017)

Cancer type (N)	TMB and response relationship	Assay type	Treatment Strategy	Comments	References
	All evaluable patients: RR 13% vs 15%; OS = HR 0.65 (95% CI 0.38-1.12); PFS 0.98 (95%CI 0.63-1.53)			Higher TMB was an independent predictor of improved RR, PFS but not OS	
NSCLC (444 pts)	*TMB high (≥20 mut/Mb) vs Intermediate/Low (<20 mut/Mb)* Median Duration of Therapy: 7.5 vs 4.6 months (p=0.001) Median OS: not reached vs 10 months (p=0.10)	NGS Foundation Medicine	ICI	Design: retrospective analysis of Flatiron Health Database Drug: nivolumab Higher TMB was predictive of benefit in the multivariable analysis	(Singal et al., 2017)
NSCLC (75 pts)	*TMB above (>158 mutations) vs. below median (≤158 mutations)* RR: 51% vs. 13% (p=0.0005) DCB: 65% vs. 34% (p=0.011) PFS: 17.1 vs 3.7 months HR 0.41, (95%CI, 0.23-0.73; p=0.0024)	WES	ICI+ICI	Design: exploratory analysis of single phase II trial (CheckMate 012) (NCT01454102). Drug: ipilimumab plus nivolumab TMB and PD-L1 were demonstrated to present independent predictive value four outcome	(Hellmann et al., 2018c)
NSCLC (299 pts)	*Ipilimumab+nivolumab vs chemotherapy comparison* High TMB (≥10 mut/Mb): RR=45.3% vs 26.9%; PFS, HR 0.58 (95% CI 0.41-0.81, p<0.001) Low TMB (<10 mut/Mb): PFS, HR 1.07 (95% CI 0.84-1.15)	NGS Foundation Medicine	ICI+ICI	Design: exploratory analysis of randomized phase III (CheckMate 227) (NCT02477826). Drug: ipilimumab plus nivolumab No correlation between TMB and PD-L1 expression level	(Hellmann et al., 2018b)
NSCLC (240 pts)	*TMB above median (>7.4 mut/Mb) vs below median* DCB rate: 38.6% vs. 25.1% (p=0.009) PFS: HR 1.38 p=0.024	MSK Impact NGS	ICI	Design: retrospective analysis of single institutional data Drugs: PD-1/PD-L1 inhibitors Estimates of TMB correlated well with NGS in 49 patients. No correlation was detected between TMB and PD-1 expression	(Rizvi et al., 2018)
NSCLC (605 pts)	*Pembrolizumab+chemotherapy versus chemotherapy* TMB >175 mut/exome: OS= HR 0.64 (95%CI 0.38-1.07); PFS = HR 0.32 (95%CI 0.21-0.51) TMB <175 mut/exome: OS= HR 0.64 (95%CI 0.42-0.97); PFS = HR 0.51 (95%CI 0.35-0.74) KEYNOTE-021 G (Pembro+CT) RR: 60.8% (95%CI 38.5-80.3) for tTMB <175 vs. 71.4% (95%CI 47.8-88.7) for tTMB ≥175 mut/exome	WES	ICI+Chemo	Design: exploratory analysis of multiple prospective trials: KEYNOTE-021 C and G (NCT02039674), 189 (NCT02578680), and 407 (NCT02775435). Drug: pembrolizumab TMB was not associated with efficacy of chemotherapy plus pembrolizumab. It is plausible that giving chemotherapy with pembrolizumab could have confounded the utility of TMB, which appears predictive with immunotherapy alone.	(Paz-Ares et al., 2019)
NSCLC (98 pts)	*TMB (≥10 mut/Mb) vs. (<10 mut/Mb)* RR: 44% vs. 12% (no p-value) PFS: 7.1 (95% CI, 3.6 to 11.3 months) vs 2.6 (95% CI, 1.4 to 5.4 months) (no p-value)	NGS Foundation Medicine	ICI	Design: expoloratory analysis of single arm prospective phase II trial (CheckMate 568). (NCT02659059). Drug: pembrolizumab RR was higher in TMB high patients regardless of PD-L1 expression	(Ready et al., 2019)
RCC (109 pts)	*TMB High (*≥64 mut) vs Low (<64/mut) Nivolumab Cohort: RR 20% vs 33% (p=0.20); PFS 1.06 (95% CI 0.64-1.75, p=0.82); OS: 0.85 (95% CI 0.43-1.67 p=0.64) Ipi+Nivo Cohort: RR 61% vs 43% (p=0.48); PFS HR 0.36 (95%CI 0.16-0.84, p=0.02); OS HR 0.71 (95%CI 0.16-3.15, p=0.65)	WES	ICI+ICI	Design: retrospective analysis of single institutional data (MSKCC) Drugs: ipilimumab+nivolumab High TMB was predictive of longer PFS only on the ipilumumab and nivolumab cohort	(Lee et al., 2019)
SCLC (211 pts)	*TMB high (*≥248 mut) *vs medium (143-247 mut) vs low (<143 mut)* RR: Nivolumab: 21.3% vs 6.8% vs 4.8% Nivo+Ipi: 46.2% vs 16% vs 22.2% PFS (1-year): Nivolumab: 21.2% vs 3.1% vs not calculable Nivo+Ipi: 30% vs 8% vs 6.2% OS (1-year): Nivolumab: 35.2% vs 26% vs 22.1% Nivo+Ipi: 64.4% vs 19.6% vs 23.4%	WES	ICI+ICI	Design: exploratory analysis of phase II trial (CheckMate 032) (NCT01928394). Drug: nivolumab or nivo+ipi High TMB predicted better efficacy for the combination compared.to nivolumab	(Hellmann et al., 2018a)

Cancer type (N)	TMB and response relationship	Assay type	Treatment Strategy	Comments	References
Urothelial carcinoma (86 pts)	*TMB upper fourth quartile (≥16 mut/Mb)* OS: higher compared to patients in quartiles 1-3 (p=0.004)	NGS Foundation Medicine	ICI	Design: exploratory analysis of prospective phase II trial (Imvigor 210) (NCT02108652) Drug: atezolizumab Higher TMB predicted better survival	(Balar et al., 2017)
Urothelial carcinoma (139 pts)	*TMB high (*≥167 mut) *vs medium (85-166 mut) vs low (<85 mut)* RR (%): 31.9 vs 17.4 vs. 10.9 (p=0.0002) PFS (months): 3.02 vs. 1.87 vs. 1.91 (p=0.005) OS (months): 11.63 vs 9.66 vs 5.72 (p=0.067)	WES	ICI	Design: exploratory analysis of single arm phase II trial (NCT02387996) Drug: nivolumab Higher TMB correlated with better RR and longer PFS	(Galsky et al., 2017a)
Urothelial carcinoma (25 pts)	*Durable clinical benefit vs no durable clinical benefit* TMB 3.24 mut/Mb vs 0.45 mut/mB (p=0.22)	WES	ICI	Design: exploratory analysis of phase II trial (Imvigor210) (NCT02108652) Drug: atezolizumab Higher TMB observed in patients with durable benefit	(Snyder et al., 2017)
Urothelial carcinoma (274 pts)	*Atezolizumab vs chemotherapy comparison* TMB high (≥median = 9.65 mut/Mb): OS= HR 0.68 (95%CI 0.51-0.90) TMB low (<median=9.65 mut/Mb): OS = HR 1.0 (95% CI 0.75-1.32)	NGS Foundation Medicine	ICI	Design: exploratory analysis of phase III randomized trial (Imvigor211) (NCT02302807) Drug: atezolizumab DDR mutations correlated with increased TMB	(Powles et al., 2018)

Abbreviations: Chemo = chemotherapy; CRC=colorectal cancer; CRPC= castration resistant prostate cancer; DCB = durable clinical benefit; DDR = DNA damage repair; HNSCC= head and neck squamous cell carcinoma; HR=hazard ratio; ICI=immune checkpoint inhibitor; ipi= ipilimumab; Mb = megabase; MSI-H= microsatellite instability-high; MSKCC= Memorial Sloan Kettering Cancer Center; mut = mutation; N = number; NGS = next generation sequencing; Nivo = nivolumab; NSCLC = non-small cell lung cancer; OS = overall survival; pts = patients; PFS = progression-free survival; RCC=renal cell carcinoma; RR = response rate; SCLC=small cell lung cancer; WES= whole exome sequencing

Table 2:

Examples of blood TMB and response to immunotherapy (checkpoint inhibitors)

Cancer type (N)	TMB and response relationship	Assay type	Treatment Strategy	Comments	References
Diverse cancers (69 pts)	*VUS > 3 vs. VUS ≤ 3* SD ≥6 months/PR/CR 45% versus 15%, respectively; *P* = 0.014 PFS: 3.84 vs 2.07 months (HR, 0.52; 95% CI, 0.31–0.87; P=0.019) OS: NR vs 10.72 (HR, 0.39; 95% CI, 0.18– 0.83; P=0.042)	NGS 54-70 genes (Guardant)	ICI	Design: retrospective analysis, single institutional data Drugs: multiple checkpoint inhibitors Of the 69 patients, 20 (29%) presented >3 VUS and 49 (71%) *≤ 3 VUS*	(Khagi et al., 2017)
NSCLC (583 pts, validation cohort)	*Atezolizumab vs docetaxel comparison* bTMB ≥16 : PFS: HR 0.65 (0.47-0.92); OS: 0.64 (0.44-0.92) bTMB <16: PFS: HR 0.98 (0.80-1.20); OS: 0.65 (0.52-0.81)	NGS 1.1 Mb (Foundation Medicine panel)	ICI	Design: exploratory analysis of prospective randomized phase II (POPLAR NCT01903993) and phase III trial (OAK NCT02008227_ Drug: atezolizumab Correlation with tTMB by Foundation Medicine assay was demonstrated (Spearman correlation 0.93).	(Gandara et al., 2018)
NSCLC (119 pts)	*bTMB ≥16 vs. <16* ORR: 28.6% vs 4.4% (P=0.0002) PFS: 5.0 vs 3.5 months (HR 0.80 90% CI 0.54-1.18) OS: 23.9 vs. 13.4 months (HR 0.66 90% CI 0.40-1.10)	NGS 1.1 Mb (Foundation Medicine panel)	ICI	Design: prospective analysis of B-F1RST single arm prospective trial (NCT02848651)_ Drug: atezolizumab bTMB was predictive of higher RR; no statistical significant benefits were detected for PFS or OS	(Kim et al., 2018; Socinski et al., 2019)
NSCLC (809 pts)	*Durvalumab+Tremelimuab vs. CT* bTMB ≥20mut/Mb: PFS 4.2 vs 4.4 months (HR 0.53 95% CI 0.34-0.81); OS 21.9 vs 10 months (HR 0.49 [95% CI 0.32-0.74) bTMB <20mut/Mb: PFS 2.0 vs 5.0 months (HR 1.55 95% CI 1.23-1.94); OS 8.5 vs 11.6 months (HR 1.16 95% CI 0.93-1.45)	Guardant OMNI (Guardant Health)	ICI+ICI	Design: exploratory analysis of prospective randomized phase III trial (MYSTIC NCT02453282) Drugs: durvalumab + tremelimumab bTMB was correlated with tTMB (Spearman's correlation = 0.6) Cut-points for dichotomization of TMB may be made to optimize positive or negative predictive value.	(Rizvi et al., 2020)
NSCLC (98 pts)	*bTMB ≥6 vs. <6* PFS: NR vs 2.9 months, (HR 0.39; 95% CI, 0.18-0.84; P = 0.01) RR: 39.3% vs. 9.1% (P=0.02)	NGS 150 genes (NCC-GP150)	ICI	Design: retrospective analysis of multi-institutional series_ Drugs: multiple PD-1/PD-L1 inhibitors The authors demonstrated a significant correlation between bTMB vs tissue TMB in a virtual comparison to TCGA (r^2=0.91) and with matched tTMB calculated by WES (Spearman correlation = 0.62)	(Wang et al., 2019)
NSCLC (66 pts)	*bTMB ≥16 vs. <16* *ORR: 28.6% vs 4.4% (P=0.0002)* *PFS: 14.1 vs 4.7 months (HR 0.30 95% CI 0.16-0.60, p<0.001)* *OS: not reached vs. 8.8 months (HR 0.48 90% CI 0.22-1.03; p=0.061)*	Guardant OMNI (Guardant Health)	ICI	Design: observational prospective cohort of single institutional data (NCT03047616) Drug: pembrolizumab bTMB>16 mut/Mb is associated with improved PFS; *STK11/ KEAP1/PTEN* and *ERBB2* mutations were common in unresponsive patients	(Aggarwal et al., 2020)

Abbreviations: bTMB = blood tumor mutational burden; CR = complete response; CT=chemotherapy; HR=hazard ratio; ICI=immune checkpoint inhibitor; mb = megabase; N = number; NGS = next generation sequencing; NR=not reached; NSCLC=non-small cell lung cancer; ORR = objective response rate; PFS = progression-free survival; PR = partial response; pts = patients; RR = response rate; SD = stable disease, tTMB=tissue tumor mutational burden; VUS = variant of unknown significance; WES = whole exome sequencing

Table 3 –

Examples of biomarkers/genomic changes other than TMB that may influence the response to checkpoint blockade

Gene/Protein	Comments	References
Possible association with increased benefit		
APOBEC-related mutagenesis	APOBEC-related mutagenesis increases neo-peptide hydrophobicity, and, thus immunogenicity. APOBEC signature correlated with responses to immunotherapy independently of TMB.	(Boichard et al., 2019)
ARID1A	*ARID1A* alterations (part of the SWI/SNF chromatin remodeling complex) function as a biomarker for longer progression-free survival after anti-PD-1/PD-L1 immunotherapy	(Okamura et al., 2020)
CDK12	*CDK12* biallelic inactivating mutations is associated with genomic instability, including increased gene fusions. Most of the genomic alterations in these tumors are not captured by current TMB estimation, but lead to increased neo-antigen burden and T cell infiltration, suggesting benefit from check-point inhibition.	(Wu et al., 2018)
DDR genes *	Alterations in DDR genes are associated with increased TMB and tumor infiltration by T cells. Some series demonstrated higher response rate and longer survival during therapy with PD-1/PD-L1 in the presence of DDR alterations. DNA damage is also associated with cellular stress that may trigger immune innate mechanisms.	(Teo et al., 2018) (Chae et al., 2019)
KRAS	In NSCLC the presence of KRAS mutations correlates with an inflammatory microenvironment, and may indicate patients that will benefit from checkpoint-inhibitors in monotherapy.	(Liu et al., 2020a).
MHC1 presentation	Poor presentation of driver mutation neoantigens by MHC-I may explain why some tumors (even with a high TMB) do not respond to checkpoint blockade.	(Goodman et al., 2020)
MLH1, MSH2, MSH6, PMS2	Loss of function of mismatch repair genes leads to microsatellite instability and a higher number of somatic mutations. These alterations were predictive of higher responses to checkpoint inhibitors.(Le et al., 2017)	(Le et al., 2017)
PBRM1 loss	*PBRM1* (part of the SWI/SNF chromatin remodeling complex) loss is associated with T-cell mediated tumor killing; series of patients with renal cell carcinoma showed higher benefit from ICIs in the presence of *PBRM1* loss	(Miao et al., 2018)
PD-1 expression	Frequency of PD-$1^{+}CD8^{+}$ T cells relative to that of PD-1^{+} regulatory T (T_{reg}) cells in the tumor microenvironment can predict the clinical efficacy of anti-PD1 therapies	(Kumagai et al., 2020)
PD-L1 amplification	PD-L1 (*CD274*) amplification may be present ~0.7% of solid tumors; most PD-L1 amplified tumors demonstrated low to intermediate TMB. Clinical responses to checkpoint ICIs are frequent (66.7%) and independent of TMB in solid tumors; however, only a small number of such patients have been treated. PD-L1 amplification is also a hallmark of Hodgkin disease, which is highly responsive to checkpoint blockade.	(Goodman et al., 2018)
PD-L1 expression by immunohistochemistry	PD-L1 overexpression is an indicator of intra-tumoral inflammatory environment. Expression levels are independent of TMB. PD-L1 expression on tumor or immune cells may be important.	(Ott et al., 2019) (Patel and Kurzrock, 2015) (Liu et al., 2020b) (Gonzalez-Ericsson et al., 2020)
POLD1, POLE	Loss-of-function mutations in *POLD1* and *POLE* correlate with increased immune infiltration and a hyper-mutated phenotype, and predict responses to anti-PD-L1 therapy.	(Budczies et al., 2018) (Wang et al., 2018)
SERPINB3, SERPINB4 mutations	Serpin mutations were associated with higher clinical benefit rates and longer survival in melanoma patients treated with anti-CTLA4 therapy. This possible positive predictive effect was independent of TMB.	(Riaz et al., 2016)
SMARCA4	*SMARCA4* (part of the SWI/SNF chromatin remodeling complex) alterations are correlated with ICI response in small cell ovarian cancer hypercalcemic type and anecdotally in other tumors	(Tischkowitz et al., 2020)
Tumor infiltrating lymphocytes	High tumor infiltrating lymphocytes have been associated with checkpoint inhibitor responsiveness	(Gonzalez-Ericsson et al., 2020)

Gene/Protein	Comments	References
Possible association with resistance		
B2M inactivating mutations	*B2M* truncating mutations lead to loss of surface expression of MHC I and resistance to PD-1 blockade.	(Zaretsky et al., 2016)
JAK1/2 mutations	*JAK1/2* loss of function mutations can lead to acquired and primary resistance to anti-PD-1 therapy, even in the setting of high TMB.	(Shin et al., 2017)
KEAP1 alterations	Associated with worse outcome after ICI. Not clear if these alterations are prognostic or predictive	(Chen et al., 2020; Skoulidis and Heymach, 2019)
STK11/LKB1	Associated with worse outcome after ICI. Not clear if these alterations are prognostic or predictive	(Skoulidis et al., 2018)
Possible association with hyper-progression		
MDM2/MDM4 and *EGFR*	*MDM2/MDM4* amplifications and *EGFR* aberrations were associated with hyperprogression in patients treated with checkpoint inhibitors. This association was independent of TMB.	(Kato et al., 2017) (Singavi et al., 2017)

* DDR genes definitions are variable and commonly includes *BRCA1, BRCA2, ATM, CHECK2, PALBB2, ATM, ERCC2, FANCA*

Abbreviations: B2M: Beta-2-microglobulin, DDR: DNA damage repair; ICI= immune checkpoint inhibitor; MHC: major histocompatibility complex