Review Article

Review of Immunogenomics and the Role of Tumor Mutational Burden as a Biomarker for Immunotherapy Response

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Abstract

Immune checkpoint inhibitors benefit a proportion of patients with cancer, but not all patients nor all histologies will respond to immunotherapy. Therefore, predictive biomarkers are needed. In this review, we outline the ways that lead to hypermutated tumors as well as the potential predictive role of tumor mutational burden (TMB). Findings in selected cancer types suggest that TMB may predict clinical response to immunotherapy, and recently even a prognostic role has been suggested for TMB. An association between high mutational load and clinical benefit was observed in various tumor types; however, it is unclear whether TMB is a strong predictive marker of clinical benefit across all cancers. For that reason, there are still several questions regarding the role of TMB as an immunotherapy biomarker, such as the best measurement technique, the most adequate cutoff, or even whether TMB will be useful for any kind of cancer. We have performed an extensive bibliography research using PubMed with keys words: immunotherapy, tumor mutational load, TMB, immunotherapy biomarkers, and immunotherapy response. In conclusion, TMB has been demonstrated to be a useful biomarker for immunotherapy selection across some cancer types; however, further validation studies are required.

Keywords: *Cancer, immunotherapy, tumor mutational burden, tumor mutational load*

Introduction

Immunotherapy has expanded the armamentarium to lead the fight against cancer in the last decade.[1] One of the main characteristics of tumors resides in the ability to remain hidden from the patient's immune system, thus evading the main mechanism of defense against the appearance of malignant cells. The use of therapies that reactivate the immune response in the tumor environment is able to produce a long treatment response in approximately 20% of cases in some types of tumors such as melanoma, lung cancer, renal and urothelial carcinomas, or even hypermutated colorectal cancer (CRC).[2,3] However, there must be a parallel effort to identify biomarkers to predict which tumors will respond to immunotherapy.[4,5] One of these emerging biomarkers is the tumor mutational burden (TMB).[5-7] The TMB is obtained by ascertaining the mutational profile of tumors, and the number of mutations that will generate changes in the proteins encoded by these genes.[8] If these proteins were expressed by the tumor cell, such mutations could eventually be identified as "neoantigens"[9] by the immune system which, when reactivated pharmacologically, will attack the tumor and control its growth. Thus, somatic mutations in tumor DNA can give rise to neoantigens (mutation-derived antigens), that are recognized and targeted by immune system cells. In addition, we have evidence that the presence of immune cells in the tumoral microenvironment is of capital importance to obtain an immune response. The TMB is calculated from genomic tests, based on the next-generation sequencing (NGS) of a large number of tumor DNA genes. However, the optimal cutoff value for TMB is still unclear. Other clinical routine tests are carried out to obtain gene expression profiles (RNA sequencing or microarrays) and surface protein expression (immunohistochemistry), which define what genes are expressed in tumors, what cellular immune populations are present in the tumor microenvironment, or to characterize potential neoantigens.

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In this context, we can use sequenced data of the major histocompatibility complex (MHC) to improve the prediction of which neoantigens will activate the immune system more efficiently. It is clear that there is a critical needed of predictive biomarkers to select patients more efficiently.[10] In this review, we outline the different ways that lead to hypermutated tumors as well as the potential predictive role of TMB. We review available data to date studying TMB as a biomarker for response to immunotherapy in several tumor types as well as data regarding how neoantigens impact in the predictive role of TMB. We included English-only papers from 2000 to 2019 that include TMB as a biomarker in clinical trials using immunotherapy as well as those papers referring how neoantigens interact with immunotherapy drug response.

Tumor Mutational Load: A Potential Predictive Marker for Programmed Death-1/Programmed Death-Ligand-1 Inhibitors

The relationship among the genomic landscape of the tumor, the mutational load and the benefit from treatment with immune modulators checkpoint inhibitors remains obscure due to its complexity, although the concepts are quite well established. The nexus between tumor mutations and the immune system could be described as follows: tumors with mutated proteins or neoantigens, which are displayed on the surface of tumors cells, enable the recognition by the host's immune systems. Mutated proteins lead to the translation of novel peptide epitopes, or neoepitopes, which are presented by the MHC on the surface of malignant cells. Theoretically, the presence of neoantigens[11] enhances the immunogenicity of the tumor by eliciting T-cell repertoires that recognize these antigens as "foreign" and infiltrate the tumor microenvironment (TME). It is predicted that immunotherapies may augment this innate anti-tumor response.[12] TMB, nonsynonymous mutation burden, or tumor mutation load is defined as the total number of mutations per coding area of a tumor genome. Unlike other markers, such as protein-based biomarkers, TMB is a quantitative measure. Recent advances in sequencing technology allow for whole exome interrogation and qualitative calculation of the total number of mutations per coding region or total mutation burden in individual cases. Tumors that have higher levels of TMB are believed to express more neoantigens that may allow for a more robust immune response and therefore a more durable response to immunotherapy. Because the immune system relies on a sufficient number of neoantigens to appropriately respond, the number of somatic mutations is, in effect, acting as a proxy for determining the number of neoantigens per tumor. TMB provides a quantitative measure that enables the clinician to make decisions, such as the selection of immunotherapies or enrollment in clinical trials. Theoretically, tumors with high mutation numbers will also have high neoantigen load. Although not all somatic mutations can give rise to neoepitopes and not all may serve as neoantigens, tumors with higher TMB are considered to produce higher amounts of neoantigens, which is a more robust tumor-associated inflammatory reaction.[13,14] Thus, high levels of TMB are associated with a number of causes, such as exposure to carcinogens and mutagens, mutations in the DNA repair pathway, and microsatellite instability (MSI). In this context, the use of NGS allowed a deeper understanding of the molecular biology of cancer. Pan-Cancer analysis showed some similarities and differences among tumors.[15] One of these is the number of mutations per tumor and tumor type. There is large variability in mutation load within tumor types, ranging from just a few to thousands of mutations per megabase (Mb). These differences have been described in lower-grade and pediatric malignancies which tend to have the lowest mutation load, while epithelial cancers associated with environmental DNA damage are most highly mutated. The highest mutational rate is observed in melanoma and in non-small cell lung cancer (NSCLC)[16-19] where mutations are known to be secondary to ultraviolet (UV) light and tobacco smoke exposure, respectively. In patients with NSCLC, never-smokers tend to have less somatic mutations than smokers, who may have ten-fold more mutations. In addition to exposure to mutagens such as UV light and tobacco smoke, abnormal activity in several cellular pathways, including DNA damage repair and replication, can increase the overall rate of somatic mutations in tumors. Pediatric malignancies had a lower number of mutations than adult malignancies, and disease types common in pediatric patients such as leukemia, lymphoma, and neuroblastoma had a low number of mutations, as do sarcomas. Still, there are cases with high mutational load across nearly every cancer type.[20] Retrospective data indicate that a high mutational load correlates with a sustained clinical benefit from cytotoxic T-lymphocyte antigen-4 (CTLA-4)[4] or programmed death-1/programmed death-ligand-1 (PD1/PDL1)[5] blockade. There are additional cancer types with high TMB, such as skin squamous cell carcinoma, skin basal cell carcinoma, small-cell lung cancer, undifferentiated carcinoma, and diffuse large B-cell lymphoma. Still, a high load alone does not seem sufficient to produce clinical benefit in all cases, because there are tumors with a high mutational burden that did not respond to immunotherapy. On the contrary, relatively low median rates of somatic mutations are observed in tumor types that exhibit limited responses to anti-PD-1/PD-L1 agents such as CRC, ovarian carcinoma, and esophageal and prostate cancer.[21,22]

Increased Number of Mutations in Cancer

The prevalence of somatic mutations among neoplasm ranges from 0.01 per Mb to more than 400 mutations per Mb. A high tumor mutation load is associated with mutations in genes for DNA mismatch repair pathways (MMR)

such as melanocyte-stimulating hormone (MSH) 2 and 6, MutL homolog 1 (MLH1), postmeiotic segregation increased 2 protein (PMS2), or MSI. The basis of microsatellites instability is the inactivation of some of the genes that are involved in DNA replication disruptions repairment, leading to an accumulation of errors in DNA and producing mutations by insertions or deletions. The vast majority of tumors with MSI also had high TMB. However, the converse is not true; only 16% of cases with high TMB are MSI-High. Many other causes of somatic hypermutation exist other than MSI, such as mutations in the DNA polymerases encoded by polymerase epsilon (POLE) or polymerase delta (POLD1), exposure to external mutagens (cigarette smoke and UV radiation), and endogenous mutagens (reactive oxygen species).[23-25] Consequently, in gastrointestinal cancers[26] such as in CRC, stomach adenocarcinoma, duodenum adenocarcinoma, and small intestine adenocarcinoma, high MSI and high TMB almost always co-occur, pointing to MSI as the main cause of accumulation of mutations in these tumors. In melanoma, squamous cell carcinoma, and NSCLC, high TMB is fairly common, but high MSI rare. In contrast to tobacco-induced NSCLC, generally characterized by high TMB, patients with specific genotypes including epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) rearrangements, ROS proto-oncogene 1 (ROS1), (proto-oncogene B-Raf) or RET (ret proto-oncogene) mutations have consistently demonstrated a lack of response to immune checkpoint inhibitors.[27] In NSCLC, the presence of mutations in oncogenic driver genes such as EGFR, ALK, ROS1, RET fusions, or cMET exon 14 skipping correlates with lower TMB,[28] as did BRAF and NRAS mutations in melanoma. A recently published retrospective analysis of 28 advanced adenocarcinoma patients with EGFR mutations (n = 22) or ALK rearrangements (n = 6) treated with PD-L1 inhibitors demonstrated response rate of only 5% and 0%, respectively.[29] However, in patients with metastatic NSCLC without ALK or EGFR alterations and with high PD-L1 (>50%) expression, the response rate was 44.8% for those patients who received pembrolizumab (anti-PD-1).[30] Nevertheless, TMB does not explain all antitumor responses seen with PD1/PDL1 inhibitors. A notable outlier is renal clear-cell carcinoma (RCCC), which has a relatively low mutational burden (around 10 times lower than melanoma) showing a 25% response rate with nivolumab.[31] Although relatively low TMB in RCCC, this high response rate could be justified because of the presence of a high number of single nucleotide variants (SNV). Recent data show that indels can produce many more neoantigens that SNV, and the response of RCC to PD1 inhibitors may be explained by a large load of indels, although the total number of somatic mutations may remain intermediate or low. However, there are some exceptions, some viral diseases, for example, may upregulate specific genes such as APOBEC (responsible for mRNA editing) and could also create immunogenic neoantigens in the absence of high TMB. These include polyomavirus-positive Merkel cell carcinoma and Hodgkin's lymphoma. Furthermore, other biological mechanisms (e.g., PD-L1 amplification) could contribute to immunotherapy response.

The Road to High Tumor Mutational Burden: Frequent Causes of Hypermutated Tumors

DNA MMR is a key mechanism for identifying and repairing erroneous misincorporation, deletion, and insertion of bases that might occur during DNA replication and recombination. These mutations could affect important growth and regulatory genes with repeat sequences. MMR deficiency can cause insertions, deletions, transitions, and transversions, leading to dysfunction in key tumor suppressor genes. The core DNA mismatch repair protein complex is composed of two cooperative dimers: the PMS2 (PMS1 Homolog 2) protein dimerizes with MLH1 to form the complex Mut-alpha, which cooperates with the MSH2-MSH6 dimer, MutS-alpha, to repair single base pair mismatches and small insertion–deletion loops.[32,33] Perturbations in MMR gene expression, by both loss and overexpression, can be deleterious to genomic stability, and loss of function mutations in MMR pathway genes are known to correlate with high TMB in tumors. As such tumors with defective DNA repair mechanisms are more likely to benefit from immunotherapy.[25] Tumors with genetic defects in MMR pathways are known to harbor 100–1000 of somatic mutations per Mb, especially in regions of repetitive DNA, known as microsatellites (microsatellites are DNA segments with repeating sequences of 2–5 base pairs). When DNA polymerase amplifies these regions enriched in repetitive sequences it increases its error rate, which causes multiple polymorphisms. The accumulation of mutations in these regions of the genome is termed "microsatellite instability." Due to the accumulation of alterations in these regions, MMR are denominated with MSI.[34] Tumors that frequently present with MSI are thyroid (63%), endometrium (22%), gastric (22%), hepatocarcinoma (15%), colorectal (13%), and skin (melanoma 11%).[35] Alterations in MMR genes can be germline (heritable) or somatic (spontaneous). Patients with MSI due to germline mutations in one of the MMR genes are defined as having Lynch syndrome, which has a particular phenotype. Lynch syndrome is an autosomal dominant condition characterized by an elevated risk for cancers of the ovaries, kidneys, bladder, stomach, small bowel, bile ducts, and brain, with the biggest increase in risk for endometrial cancer and CRC. Sporadic MSI usually arises from epigenetic silencing of the MLH1 promoter, often from a global increase in CpG (cytosine-phosphate-guanine; stretches of DNA 500–1500 bp long with a cytosine-guanine: guanine-cytosine ratio of more than 0.6) island methylation and frequently associated with a somatic BRAF p.V600E mutation.[36] Based on this, to

differentiate Lynch syndrome and MSI, one should test for mutations in MLH1, MSH2, MSH6, PMS2, their protein expression, the presence of MSI regions, methylation of MLH1 promoter, and BRAF mutation. Mutations in MMR protein expression (loss of MLH1, MSH2, MSH6, and PMS2) could be established by immunochemistry, which has 91% of sensitivity and 87% of specificity. Still, more than 30% of MLH1 mutations are missense mutations and therefore would not alter protein expression. The sensitivity for detection of DNA MMR increases when all four MMR proteins are tested because of the heterodimer structure of MLH1/PMS2 and MSH2/MSH6 (when either MLH1 or MSH2 is functionally lost, the heterodimer becomes unstable and PMS2 or MSH6, respectively, is degraded). Polymerase chain reaction (PCR), with 97% sensitivity and 95% specificity, is another way to test MMR. The Bethesda guidelines[37] recommended a reference panel of five microsatellites (when at least two of the five microsatellite loci are abnormal, the tumor is defined as MSI-high; those with <30%–40% [one of five] are considered MSI-low, and those without instability are MSS). However, since there are many microsatellites throughout the genome, their applicability to all tumor types is unclear. Concordance between immunohistochemistry and PCR is around 97%. Discordance can occur due to truncation mutations with retained antigenicity and protein expression, although the protein is not functional. As a consequence, there is MSI, but the protein is still expressed. Another test from Promega Corporation (Madison, Wisconsin, USA), called the "MSI Analysis System," uses a multiple PCR system marked with fluorescence. MMR could be also determined by NGS: testing multiple microsatellite loci allows the calculation of fraction on unstable microsatellite loci. This technique has a 97% sensitivity and 98% specificity (similar to PCR).[38-40] A commercial assay such as FoundationOne (Cambridge, Massachusetts, USA)[39] seems to be able to reproduce similar results as PCR. Actually, there are several assays in development to evaluate MSI in circulating-free DNA (cfDNA) (liquid biopsies) based on the NGS. Another mechanism to develop high TMB could be through BRCA 1/2 alterations.[41] Given the central role of BRCA1 and 2 in homologous recombination-mediated DNA repair and the maintenance of genomic integrity, BRCA1-mutated breast tumors have a high mutational burden. These tumors also are characterized by a strong lymphocyte infiltration.[42] These cases are also associated with favorable outcome. BRCA-1/2-mutated high-grade serous ovarian cancers with high mutational load have been shown to harbor increased numbers of neoantigens compared with tumors proficient in homologous recombination and are associated with improved overall survival (OS). Despite all these, initial results of the response to immune checkpoint inhibitors; have not been as encouraging as in other settings.[43] Dysfunction in polymerases could also lead to more immunogenic tumors.[44] DNA replication is another key pathway in which defects can lead to an increased somatic mutation rate. Recognition and removal of errors during replication are critical functions of DNA polymerases responsible for the majority of nuclear DNA replication, and POLD1 and POLE are involved in the removal of errors during lagging- and leading-strand replication, respectively. Tumors with somatic point mutations in the exonuclease domain of POLE or POLD1 have some of the highest mutational burdens identified to date. Therefore, mutations in these genes can result in hypermutation and tumorigenesis.[45] The loss of TP53 DNA damage checkpoint activity, by somatic mutation, copy number loss or epigenetic silencing, increases DNA damage tolerance and can also be associated with increased mutation frequency.

Resistance to Immunotherapy in Hypermutated Tumors

Some mechanisms of resistance to checkpoint inhibitors have been described, including upregulation of alternate immune checkpoints, loss of human leukocyte antigen (HLA) haplotypes, or somatic mutations in HLA, beta-2-microglobulin or JAK1/JAK2 genes.[46] These mutations are involved in interferon receptor signaling and antigen presentation pathways.[47] Mutation processing and antigen presentation could also be disrupted by the loss of mutation-associated neoantigens in tumors as mechanisms of acquired resistance to anti-PD-1 or anti-PD-1/antiCTLA-4 antibodies. Neoantigen loss can occur through the elimination of tumor subclones or through the deletion of chromosomal regions containing truncal alterations and is associated with changes in T-cell receptor clonality.[48] In these cases, changes in the mutation landscape of the tumor can possibly predict the development of acquired resistance to treatment with immune checkpoint inhibitors. Interestingly, it has been published that loss of function of an RNA-editing enzyme, *ADAR1*, in tumor cells profoundly sensitizes tumors to immunotherapy and overcomes resistance to the immune checkpoint blockade with anti-PD1 agents.[49]

Measuring Tumor Mutational Burden in Clinical Practice

Measuring mutational load can act as a proxy for determining the number of neoantigens per tumor, and may offer a potential addition to the current targeted therapy landscape to select those patients who could obtain benefit from immunotherapy.[28] There is a growing body of clinical research demonstrating the potential benefits of TMB as a diagnostic marker in terms of accuracy, sensitivity, and reproducibility. Most importantly, it provides a quantitative measure that can be used to better inform treatment decisions.[35,39,40,43,47,48] TMB has not only a predictive role to immunotherapy response but also a prognostic role, as a recent study has shown.[50] Initial studies of TMB were using whole exome sequencing (WES) in a

retrospective approach. WES is expensive, time-consuming and hard-working technique, especially due to its bioinformatics requirements, and, therefore, difficult to incorporate into clinical practice.[28] For these reasons, efforts are underway to develop methods to accurately estimate total mutation load from widely available NGS gene panels. It has been shown that the mutation load of the whole genome can be inferred from sequencing a smaller panel of just a few hundred genes if sufficient genome is analyzed (1 Mb).[38,51] Table 1 summarizes the most relevant TMB assays. There are more than 10 tests in development that aim to analyze TMB. One of those is FoundationOne, a validated, NGS assay that targets approximately 1.1 Mb of coding genome that correlates with WES to accurately assess TMB. The targeted sequencing platform characterizes base substitutions, short insertions, deletions, copy number alterations, and selected fusions in more than 300 cancer-related genes. TMB levels are divided into three groups: low (1–5 mutations/Mb), intermediate (6–19 mutations/Mb), and high (≥20 mutations/Mb).[52] Because cancers are not static and can acquire mutations as they evolve, liquid biopsies and the analyses of cfDNA seem to accurately reflect the current mutational burden of a tumor. Another key question to the quantification of TMB and its use in the clinic is the definition of reliable mutation thresholds that identify high-mutation from low-mutation samples, enabling the prediction of response to immunotherapy and its clinical application. Recently, researchers examined the association between mutational burden and response to immune checkpoint therapy in several cancer types and were able to define a mutational burden threshold for eight of the 33 solid cancers tested, predicting response to an immune checkpoint blockade.[53] By using mutation and gene expression data from The Cancer Genome Atlas, they defined a gene expression signature of immune checkpoint–activating mutation and used this to define the threshold for TMB. In other tumors, such as triple negative breast cancer, RCC, or Merkel cell carcinoma, this was not possible, possibly because factors other than tumor mutational load are probably responsible for immune checkpoint activation (genomic rearrangements and viral etiology). As seen, neither TMB nor neoantigen burden may not be the only predictive factors for response to immunotherapy. Nevertheless, and despite the great expectancy TMB has raised, its value still needs to be validated. In fact, in June 2018, the United States food and drug administration (FDA) accepted first-line nivolumab (Opdivo) and ipilimumab (Yervoy) combination for advanced NSCLC for supplemental biologics license application (sBLA) based on data from the phase III CheckMate-227 trial (NCT02477826). This trial showed superior 1-year progression-free survival for patients with high TMB assigned to the immunotherapy combination group compared with those assigned to chemotherapy, irrespective of PD-L1 status.[54] However, the updated data in patients with low TMB showed no differences for OS with immunotherapy combination versus chemotherapy, comparable to that observed in patients with high TMB. For that reason, Bristol-Myers Squibb (BMS) has withdrawn its sBLA application, waiting for the final data, in order to clarify the relationship between TMB and PDL1 and the impact in OS of nivolumab and ipilimumab in first-line NSCLC. Given this evidence, it is clear that predictive biomarkers are needed for a more accurate patient selection. Moreover, TMB will not replace other biomarkers such as PDL1 expression but will probably complement them. The evidence available suggests that in selected tumor types, TMB may predict clinical response to immunotherapy. Globally, we can assume that higher somatic TMB is associated with better OS, but that statement is not true for all types of histologies, as a trend toward poorer survival has been reported in patients with hypermutant glioblastoma multiforme.[50,55] It seems that for immunotherapy, an ideal biomarker would be a multicomponent predictive biomarker system that combines tumor mutation load with other parameters, such as clonality, gene, and protein expression (immunosuppressive factors in the tumor microenvironment), neoantigens, MSI status, and immune targets (e.g. CD40, Lag3, and IDO). A biomarker like this should also interrogate the presence of an adequate antitumor T-cell repertoire and intact antigen presentation machinery for processing and presentation.

Summary

Genetic alterations in tumors include nonsynonymous mutations, synonymous mutations, insertions, and deletions,

Table 1: Characteristics of the main tumor mutational burden assays

Parameter	WES	FM NGS	MSKCC
Number of genes	22.000 gene coding regions	324 cancer-related genes	468 cancer-related genes
Type of mutation	Coding missense mutations	Coding missense and indel mutations	Coding missense mutations
Germline mutations	Identified and removed	Not sequenced	Identified from blood samples and removed
Capture region (tumor DNA) (Mb)	30	0.8	1.22
FDA approval	No	Yes	Yes
Price	High	Low	Low

WES: Whole exome sequencing, FM-NGS: Foundation medicine next-generation sequencing, MSKCC: Memorial Sloan Kettering Cancer Center, FDA: United States food and drug administration

as well as copy number gains and losses. Those alterations will lead to the generation of neopeptides, however, only a minority of these will be properly processed and presented in the cell surface, so only a fraction of neoantigens will be immunogenic. Moreover, not only the presence of neoantigens will impact on the immune response against tumors but also other mutations that can impair the ability of the cells to present peptides to the immune system. A complex network of relationships between TMB and the tumor biological landscape needs to be understood to improve biomarker use. Another challenge is to understand how to use TMB when specific mutations that have shown to impact response to immunotherapy appear or when immune evasion systems co-exist with high TMB. The importance of these interactions should be tested in prospective clinical trials. On the other hand, there is a well-defined TMB variation across tumor types; however, a TMB threshold has been proposed of approximate 200 nonsynonymous somatic mutations by WES.[28] Several questions need to be resolved across these different platforms such as the depth of sequencing and, length of sequencing reads as well as preanalytical factors and bioinformatics data interpretation or whether to include germline mutations in the analyses. Although these questions remain unclear, it is clear that TMB is a useful biomarker across several tumor types to predict which patients will benefit from immunotherapy treatments.

Conclusion

TMB has been demonstrated to be a useful biomarker for immunotherapy treatments across several cancer types. Although there are several questions that remain unclear regarding the best platform to define TMB, WES seems to be the best technique because of its whole exome measurement, whereas NGS is the most available platform in daily clinic. Future investigations are needed to standardize performance of NGS methods and the interpretation of results.

Despite the variation of TMB described across tumor types, a TMB threshold has been proposed, but it is known that there will be other biological factors that impact immunotherapy response. The relationship between immunotherapy response and TMB will gather not only high immunogenic neoantigens but also other mutations and microenvironment features that need a deeper understanding. Future clinical trials are needed to give a prospective view of the interaction of TMB with other biomarkers such as PDL1 expression or gene expression profile.

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