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REVIEW

Tumour mutational burden: an overview for pathologists

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Summary

Cancer immunotherapy holds great promise and has shown durable responses in many patients; however, these responses are not uniform in all patients or all tumour streams. There is an ongoing clinical need for objective diagnostic biomarkers to identify patients that will respond to immunotherapies.

Tumour mutational burden (TMB) is a diagnostic biomarker that can stratify cancer patients for response to immune checkpoint inhibitor therapies. It is commonly defined as the average number of somatic mutations per megabase in a tumour exome.

Here we describe the TMB biomarker, how it is determined, its underlying molecular basis, the relationship to neoantigens and the issues around its clinical use. This overview is directed toward practising pathologists wishing to be informed of this predictive biomarker.

Key words: Tumour mutational burden; biomarker; cancer genomics; immune checkpoint blockade; neoantigen prediction; clinical sequencing; precision oncology; molecular diagnostics.

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INTRODUCTION

Harnessing the immune system for cancer therapies is a rapidly developing area of research that has shown great clinical promise. One class of immunotherapy in particular, the immune checkpoint inhibitors (ICI), have demonstrated durable responses in solid tumours and significant improvements in relapse-free and overall survival.[1,2] However, as these responses are not uniform in all patients or all tumour types,[3,4] predictive biomarkers have been employed to identify patients likely to respond. Thus, pathologists have become familiar with the use of PD-L1 and the assessment of the mismatch repair (MMR) proteins using immunohistochemistry (IHC) in lieu of identifying microsatellite instability (MSI) that enrich for patients likely to benefit from immunotherapies.[5] However, since these biomarkers are recognised to have issues,[6] more objective immunotherapeutic predictive biomarkers are warranted.

Tumour mutational burden (TMB) is the quantity of acquired mutations in a tumour's genome and is an emerging biomarker[7] for assessing patient response to ICI. Indeed, the US Food and Drug Administration (FDA) has approved the PD-1 inhibitor pembrolizumab for patients with solid tumours lacking a satisfactory alternative treatment option, based on a single universal TMB cut-off, irrespective of histology.[8]

Here we outline the current methodologies for measuring TMB, the underlying molecular rationale for its assessment and highlight diagnostic issues that affect its measurement. This short review is a primer for practising pathologists and aims to provide information relevant to daily practice and help them understand the place of this relatively novel biomarker in cancer diagnostics.

EXISTING ICI BIOMARKERS

Anatomical pathologists will be familiar with PD-L1 as an existing predictive biomarker for response to ICI. Deficiency in the mismatch repair complex (dMMR) inferred by IHC testing for loss of expression of the key mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 is also a ICI predictive marker. Inactivation of this complex is associated with reduced DNA replication fidelity, leading to microsatellite instability (MSI) and the mutator phenotype often seen in colorectal and other cancers. Microsatellite stability itself, the gain or loss of nucleotides in microsatellite repeats scattered throughout the genome, can be measured directly by DNA fragment analysis or NGS.[9] An advantage of NGS (e.g., Illumina TruSight Oncology 500) is that a corresponding normal sample is not required and it can also directly detect loss of function mutations in MMR genes although this has not yet displaced IHC testing due to cost.

To this suite of biomarkers, we can now add TMB to provide overlapping and sometimes complementary predictors for response to ICI therapies. However, the overlap of biomarker status varies across cancer types, and in a recent study, between TMB, MSI, and PD-L1, only 0.6% of cases were positive for all three markers.[10] In a clinical trial, compared to PD-L1 expression, TMB was more significantly associated with positive response rate.[11] In another study, 83% of MSI-High samples also had high TMB but the converse did not hold, with only 16% of TMB-High samples being MSI-High.[5] The intersection of these two biomarkers was also highly dependent on cancer type. There is also some evidence that using both TMB and PD-L1 status may better predict responsiveness to ICIs than using either biomarker alone.[12]

MEASURING TMB

At its simplest, TMB is expressed as the number of acquired mutations per megabase (mut/Mb) of sequenced DNA commonly assessed by next generation sequencing (NGS) in the

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tumour genome (Fig. 1). The gold standard TMB measurement is derived from whole exome sequencing (WES) which targets the ~30 Mb of coding regions, or 1% of the genome.

In a clinical setting, WES is not currently practical in terms of constraints on overall cost of sequencing, tissue availability and turnaround times, hence smaller targeted panels that are already in widespread use have generally been used to measure TMB. However, a trade-off in measuring TMB from panels is that targeting fewer genes in smaller genomic regions leads to a reduction in the confidence of the TMB measurement.[12] In addition, the preponderance of cancer genes in panels can inflate the estimate of genome wide mutational burden as these genes are more often altered in cancer patients. Thus, a minimum size of 1Mb should be covered by any targeted panels for clinical TMB assessment.[5,13] Currently, there are over 10 diagnostic NGS panels available for determining TMB[12,14–16] and include MSK-IMPACT from Memorial Sloan Kettering (1.14Mb, 468 genes), TSO500 from Illumina[17] (1.33Mb, 523 genes) and Foundation One CDx from Foundation Medicine (0.8Mb, 324 genes), the latter which is FDA approved as a companion diagnostic for the ICI drug pembrolizumab.

These targeted panels cover between 300 and 600 cancer associated genes and are designed to identify genomic variants for treatment purposes, but also use various bioinformatic pipelines to infer a score for TMB to assess patient suitability for ICIs. Although precision oncology focusses on driver mutations for targeted therapies and ignores benign passenger mutations, both passenger and driver mutations are generally incorporated into the TMB measurement as both have the ability to generate neoantigens (see below) that have the potential to induce an immunogenic response.

It should be noted that these tests use different algorithms for analysing the NGS data, calling variants and calculating TMB. For example, some algorithms count only non-synonymous variants (protein affecting) while others use both non-synonymous and synonymous variants to calculate TMB, the synonymous variants improving variant calling accuracy. Some TMB assays include driver mutations whereas other assays exclude these by filtering out cancer hotspot mutations.[12] This filtering mitigates the effect of an over representation of cancer genes that may inflate TMB counts while also reducing the effect of different gene compositions in different panels. The calculation of TMB is also significantly affected by other bioinformatic parameters including read counting, which variant caller is used, the different handling of variant types such as indels, filtering of germline variants and annotation against alternative transcripts. This variability highlights the need for harmonising TMB calculation methods across the available panels.[5,12,14,16]

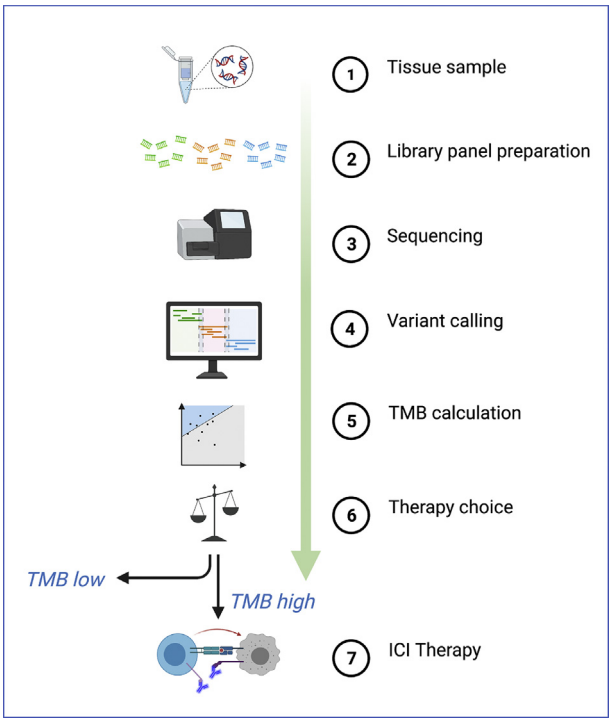

Fig. 1 TMB diagnostic workflow.

Multiple cancer genome studies have shown that somatic mutation counts range widely across different cancer types, the highest occurring in DNA repair defective tumours and mutagen driven cancers. Thus, tumours such as melanomas and lung cancers may contain ≥200 non-synonymous mutations per tumour genome while paediatric cancers contain an average of only 9.6 non-synonymous mutations per tumour genome.[18] This is reflected in TMB measurements by WES where the median TMB is approximately 10 mut/Mb for lung and skin cancers, dropping to less than one mutation per megabase for thyroid adenocarcinoma.[12]

Despite this variation, a single value of TMB (≥10 mut/Mb) has recently been approved by the FDA for the use of pembrolizumab (a PD-1 inhibitor) across all cancer types.[8] However, as above, TMB values vary widely between tumour types so that some tumours will rarely have TMB above a cut-off of 10 mut/Mb. For example, >20% of melanomas, lung tumours, stomach adenocarcinomas, transitional-cell carcinomas and uterine adenocarcinomas from 8,273 pan-cancer TCGA samples would meet this TMB cut-off.[12] However, less than 5% of breast, kidney and ovarian cancers would meet the threshold. So although responses to ICI have been documented for these 'low TMB' tumour types,[12,14] there are likely to be different enrichment cut-points in different tumour types. Thus, although the 10 mut/Mb cut-point may have been selected as a pragmatic solution, the paucity of clinical studies to validate cut-offs are likely to limit the impact of TMB application to the higher mutational burden cancers.

Unsurprisingly, low tumour purity will cause an underestimation of TMB measurement. Normal DNA from the non-neoplastic tissues will reduce the genome-wide mutation counts of tumour DNA as they become harder to detect, although it is possible to mitigate this effect by using an adjusted score (cTMB).[4] This enhanced predictor takes into account multiple attributes including tumour purity, receptor tyrosine kinase mutations, smoking related mutational signatures and HLA status to improve clinical response predictions. This type of modification may help to further refine TMB as a biomarker and improve its accuracy in predicting response to ICI response.[4]

To date most TMB assessment is derived from solid tumour tissue testing but the availability of ctDNA assays also opens the possibility of measuring TMB from liquid biopsies such as plasma, an attractive option where tissue is difficult to acquire in the relapsed or advanced setting e.g., non-small cell lung cancer[19,20] and prostate[21] cancers.

MOLECULAR BASIS FOR TMB AS A PREDICTIVE IMMUNOTHERAPY BIOMARKER

Cancer is a genetic disease driven by processes leading to an accumulation of somatic mutations on a background of any hereditary germline cancer predisposition gene alterations. These processes may originate from extrinsic factors such as mutagens (UV light, radiation or tobacco smoke) or intrinsic factors such as defects in the DNA damage response that generate characteristic mutational signatures.[22] Some tumour types tend to have specific DNA replication deficiencies such as POLE mutations in endometrial cancer[23] or MMR gene mutations in colorectal cancer,[24] while others tend to have specific mutational proclivities such as hyperactivity of the APOBEC family of deaminases in some breast and bladder cancers. These processes elevate mutation counts and give rise to specific hypermutation phenotypes such as MSI and kataegis (a pattern of hypermutation in localised genomic regions).[25]

Genetic changes in a cancer cell's DNA result in altered protein expression within the cell. This altered expression is presented on the tumour cell's surface through the usual antigen processing and surface presentation machinery.[26] Proteins are broken down into peptide fragments (~8–14 amino acids), bound to the major histocompatibility complex (MHC class I) in the endoplasmic reticulum, and are then transported to the cell surface where they are presented to the cell's microenvironment. Presented peptides from normal cellular activity will not generate an immune reaction due to the mechanism of central tolerance.[27] However, if the peptide fragment is derived from a foreign organism (such as a virus) or a mutated protein from a cancer cell, a so called neoantigen, this may be recognised by T and B cells and trigger an adaptive immune reaction against the harbouring cell. Aberrant methylation within a cancer cell can also elicit an immune response by expression of peptide sequences from quiescent germline genes not usually present in normal cells or viral genes. These types of changes can also create tumour specific antigens (TSAs) that present attractive vaccine targets for cancer therapy as they will not appear in normal cells.[28–30]

As the TMB biomarker is an aggregate measure of cancer mutational load, it acts as a surrogate for the probability that a cancer cell might present a neoantigen to the immune system. Hence, a patient with a high TMB tumour is more likely to have a primed immune system than a patient with a low TMB tumour, and this can then be activated by an ICI[31] (Fig. 2).

NEOANTIGEN ASSESSMENT AS A PREDICTIVE MARKER

Nevertheless, not all mutations have the same immunogenic potential. In light of this, one could hypothesise that cancer neoantigen assessment might be a more accurate predictor of response to immunotherapies. It is possible to predict the likely neoantigens generated by a tumour's mutations using software tools. While many of these tools use state of the art machine learning techniques, their predictive ability is constrained by the size and generality of training sets available to build a classifier. A cancer genome can generate a mutated peptide from missense and indel variants, frameshift variants, splice site variants, promoter site variants, gene fusions and post-transcriptional frameshifts (Table 1). A missense variant that results in a single peptide change may not induce a strong signal to the immune system and indeed, if the epitope is bound to the MHC with the altered peptide facing away from the T-cell receptor (TCR) then there may be no immune signal at all. Conversely, a mutated peptide in an epitope may also act as a novel anchor residue resulting in a novel epitope being presented. Generally, a simple non-synonymous variant may present fewer novel peptides than an frameshift causing indel which generates a new open reading frame potentially encoding a long novel peptide sequence. Splice site and structural variants may also create multiple novel peptides having great immunogenic potential.

To date the relative immaturity of neoantigen assessment algorithms, the nuanced types of tumour mutations, and their varied immunogenic potential, has limited the clinical utility of these tools for patient ICI stratification. Nevertheless, with newer epitope pipelines modelling peptide processing, HLA typing and peptide to MHC binding affinities,[32] and with improved databases of antigen immune environment interactions,[32,33] we are likely to see the TMB biomarker replaced with more sophisticated algorithms that incorporate emerging immunogenomic knowledge.

ISSUES WITH TMB

The universal TMB cut-off of 10 mut/Mb as a predictive biomarker of response to ICI has been criticised for not being applicable to all solid cancer types.[34] Further, as highlighted above, there is a need to harmonise the multiple diagnostic assays that measure TMB to take into account the different bioinformatic pipelines, size of targeted assays, and calibration between a targeted panel and WES.[12,14,16] Other factors which

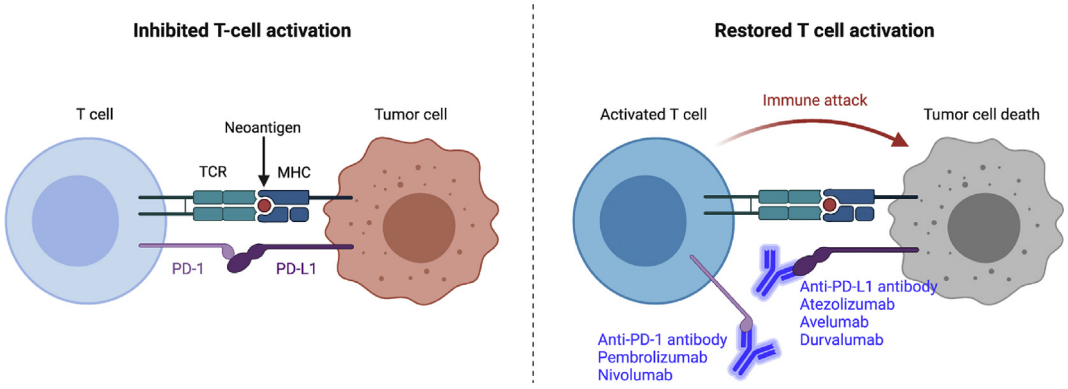

Fig. 2 T-cell activation with immunotherapy. Schematic comparing inhibited and restored T-cell activity against tumour cell using anti-PD-1 or anti-PD-L1 antibodies. MHC, major histocompatibility complex; TCR, T-cell receptor. Created with BioRender.com.

Table 1 Genomic changes impacting TMB and immunogenicity

Genomic change	Immunogenic potential?	TMB input	Typical neoantigen amino acid changes	Comments
Non-synonymous variants	Y	Y	Single	
Synonymous variants	N	Sometimes	None	May be used to improve TMB estimation although not immunogenic
Indel variants	Y	Y	Multiple	Multiple neoantigens can be created by a single indel through frameshifts
Splice site	Y	Sometimes	Multiple	
Promoter site mutations[a]	Y	N	Multiple	May cause change of protein expression levels
Gene fusions[a]	Y	N	Multiple	
Copy number variants[a]	Y	N	Multiple	Structural variants at CNV junctions may create neoantigens
Multi-nucleotide variants[a]	Y	N	Multiple	Excluded from TMB estimations[17]
Germline polymorphisms	N	N	None	Excluded from TMB estimations by public database filtering (dbSNP, gnomAD)[17]
Private polymorphisms	N	N	None	Excluded from TMB estimations by bioinformatic filtering[17]
Tumour driver variants	Y	Y	Single	Excluded from TMB estimations by bioinformatic filtering of hotspot mutations[17]
Tumour non-synonymous passenger variants	Y	Y	Single	
Non-coding variants[a]	Y[37]	N	Multiple	Results in neoantigens from aberrantly expressed transcripts including endogenous retro-elements
Endogenous retro-elements[a]	Y[37]	N	Multiple	Aberrantly methylated reading frames may be expressed

CNV, copy number variants; N, no; Y, yes; TMB, tumour mutational burden.
[a] Genomic changes not usually considered in TMB estimation.

can impact TMB measurement include patient age (TMB increases by a factor of ×2.4 between ages 10 and 90 due to the accumulation of DNA mutations from cell division replication errors),[5] microsatellite instability and mismatch repair status which may not be synonymous with high TMB. TMB algorithms that rely on public databases of genetic variation[35] to exclude germline variants from the TMB calculation are prone to over estimating TMB in individuals from minority ethnic backgrounds (which are not well represented in public databases). Algorithms that rely on germline subtraction from a matched normal sample are more robust in this regard.

Whether TMB is a good proxy for tumour immunogenicity is an open research question and the activities to develop more standardised ways of modelling cancer cell immunogenicity is an area of active research.[32,36] Future measures of TMB may integrate results from epitope prediction tools and also include neoantigens generated from non-coding regions of the genome from aberrant methylation.[36,37]

REGULATORY APPROVAL OF TUMOUR AGNOSTIC BIOMARKERS

There are implementation challenges for TMB and other novel tumour agnostic biomarkers in general. As the pathologist community knows, reimbursement for cancer genomics has been challenging and any reimbursement application for TMB as a new predictive marker would necessarily be lodged as part of a co-dependent submission, most likely with an ICI or other immunotherapy under the Public Benefits Advisory Committee assessment. It would be considered as a single biomarker in such an application, although in practice, it will be performed as part of a panel with a broader purpose such as diagnosing or identifying patients eligible for a number of different therapies. The allocation of costing and any cost offsets of the wider use of such a panel may be difficult to attribute to an individual biomarker when combined within such a complex assay.

There may be difficulty generating sufficient evidence to meet current regulatory or reimbursement thresholds for demonstration of clinical validity and clinical utility; indeed, the demonstration of clinical validity may not be possible as this will essentially be demonstrated by any observed treatment effect. Acceptance as a tumour agnostic biomarker enriching for response may be difficult when the first consideration is in the context of rare cancers or for identifying only a small proportion of those common cancers that rarely exceed the designated TMB threshold. The single arm basket study design evolved to address this issue by collating small numbers of individual tumours based on a common biomarker, acknowledging that randomisation is not always possible. In addition, the recently adopted provisional regulatory approval pathways can now accommodate efficacy endpoints that harbour greater uncertainty, such as overall response rate and duration of response. However, unresolved issues remain: payers are less likely to find data from a single arm study, with its absence of randomisation and clearly identifiable comparator, satisfactory to establish cost effectiveness. Other critical issues that need to be addressed are the cross validation of the different assays available within Australia, without which there may be uncertainties about the interchangeability of individual tests, and whether the overseas performance of these assays, in whole or in part, affects their subsidy eligibility.

CONCLUSION

Used within the scope of the validated cancer types, TMB represents a useful and clinically accessible predictive biomarker allowing for the more targeted application of ICIs in cancer therapies.[12] Further validation studies are needed to extend the application of TMB to a broader range of tumour types with specific cut-offs matched to individual tumour types.

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References

1. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J Clin Oncol* 2015; 33: 1974–82.
2. Brahmer JR, Tykodi SS, Chow LQM, *et al*. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012; 366: 2455–65.
3. Chan TA, Yarchorn M, Jaffee E, *et al*. Development of tumor mutation burden as an immunotherapy biomarker: utility for the oncology clinic. *Ann Oncol* 2019; 30: 44–56.
4. Anagnostou V, Niknafs N, Marrone K, *et al*. Multimodal genomic features predict outcome of immune checkpoint blockade in non-small-cell lung cancer. *Nat Cancer* 2020; 1: 99–111.
5. Chalmers ZR, Connelly CF, Fabrizo D, *et al*. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med* 2017; 9: 34.
6. Cooper WA, Russell PA, Cherian M, *et al*. Intra- and interobserver reproducibility assessment of PD-L1 biomarker in non-small cell lung cancer. *Clin Cancer Res* 2017; 23: 4569–77.
7. Yarchoan M, Hopkins A, Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. *N Engl J Med* 2017; 377: 2500–1.
8. Marabelle A, Fakih M, Lopez J, *et al*. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. *Lancet Oncol* 2020; 21: 1353–65.
9. Gan C, Love C, Beshay V, *et al*. Applicability of next generation sequencing technology in microsatellite instability testing. *Genes (Basel)* 2015; 6: 46–59.
10. Vanderwalde A, Spetzle D, Xiao N, Gatalica Z, Marshall J. Microsatellite instability status determined by next-generation sequencing and compared with PD-L1 and tumor mutational burden in 11,348 patients. *Cancer Med* 2018; 7: 746–56.
11. Rosenberg JE, Hoffman-Censits J, Powles T, *et al*. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet* 2016; 387: 1909–20.
12. Sha D, Zhaohui J, Budczies J, Kluck K, Stenzinger A, Sinicrope FA. Tumor mutational burden as a predictive biomarker in solid tumors. *Cancer Discov* 2020; 10: 1808–25.
13. Buchhalter I, Rempel E, Endris V, *et al*. Size matters: dissecting key parameters for panel-based tumor mutational burden analysis. *Int J Cancer* 2019; 144: 848–58.
14. Merino DM, McShane L, Fabrizio D, *et al*. Establishing guidelines to harmonize tumor mutational burden (TMB): in silico assessment of variation in TMB quantification across diagnostic platforms: phase I of the Friends of Cancer Research TMB Harmonization Project. *J Immunother Cancer* 2020; 8: e000147.
15. Endris V, Buchhalter I, Allgäuer M, *et al*. Measurement of tumor mutational burden (TMB) in routine molecular diagnostics: in silico and real-life analysis of three larger gene panels. *Intl J Cancer* 2019; 144: 2303–12.
16. Heydt C, Rehker J, Pappesch R, *et al*. Analysis of tumor mutational burden: correlation of five large gene panels with whole exome sequencing. *Sci Rep* 2020; 10: 11387.
17. Zhao C, Jiang T, Hyun J, *et al*. TruSight Oncology 500: enabling comprehensive genomic profiling and biomarker reporting with targeted sequencing. *bioRxiv* 2020; Oct 22: https://doi.org/10.1101/2020.10.21.349100.
18. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz Jr LA, Kinzler KW. Cancer genome landscapes. *Science* 2013; 339: 1546–58.
19. Chae YK, Davis AA, Agte S, *et al*. Clinical implications of circulating tumor DNA tumor mutational burden (ctDNA TMB) in non-small cell lung cancer. *Oncologist* 2019; 24: 820–8.
20. Gandara DR, Paull SM, Kowanetz M, *et al*. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. *Nat Med* 2018; 24: 1441–8.
21. Qiu P, Poehlein CH, Marton MJ, Laterza OF, Levitan D. Measuring tumor mutational burden (TMB) in plasma from mCRPC patients using two commercial NGS assays. *Sci Rep* 2019; 9: 114.
22. Alexandrov LB, Nik-Zainal S, Wedge DC, *et al*. Signatures of mutational processes in human cancer. *Nature* 2013; 500: 415–21.
23. Wang C, Gong J, Tu TY, Lee PP, Fakih M. Immune profiling of microsatellite instability-high and polymerase epsilon (POLE)-mutated metastatic colorectal tumors identifies predictors of response to anti-PD-1 therapy. *J Gastrointest Oncol* 2018; 9: 404–15.
24. Loeb LA. Human cancers express a mutator phenotype: hypothesis, origin, and consequences. *Cancer Res* 2016; 76: 2057–9.
25. Vile RG, Melcher A, Pandha H, Harrington KJ, Pulido JS. APOBEC and cancer viroimmunotherapy: thinking the unthinkable. *Clin Cancer Res* 2021; 27: 3280–90.
26. Coulie PG, Van den Eynde BJ, van der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat Rev Cancer* 2014; 14: 135–46.
27. Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* 2005; 5: 772–82.
28. Hu Z, Ott PA, Wu CJ. Towards personalized, tumour-specific, therapeutic vaccines for cancer. *Nat Rev Immunol* 2018; 18: 168–82.
29. Sahin U, Türeci Ö. Personalized vaccines for cancer immunotherapy. *Science* 2018; 359: 1355–60.
30. Sahin U, Derhovanessian E, Miller M, *et al*. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* 2017; 547: 222–6.
31. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012; 12: 252–64.
32. Richters MM, Xia H, Campbell KM, *et al*. Best practices for bioinformatic characterization of neoantigens for clinical utility. *Genome Med* 2019; 11: 56.
33. Finotello F, Rieder D, Hackl H, Trajanoski Z. Next-generation computational tools for interrogating cancer immunity. *Nat Rev Genet* 2019; 20: 724–46.
34. McGrail DJ, Pilié PG, Rashid NU, *et al*. High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types. *Ann Oncol* 2021; 32: 661–72.
35. Karczewski KJ, Francioli LC, Tiao G, *et al*. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature* 2020; 581: 434–43.
36. Minati R, Perreaul C, Thibault PA. Roadmap toward the definition of actionable tumor-specific antigens. *Front Immunol* 2020; 11: 583287.
37. Laumont CM, Vincent K, Hesnard L, *et al*. Noncoding regions are the main source of targetable tumor-specific antigens. *Sci Transl Med* 2018; 10: eaau5516.