# Challenges in bioinformatics approaches to tumor mutation burden analysis

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**Abstract.** Several immune checkpoint inhibitors (ICIs) have already been introduced into clinical practice or are in advanced phases of clinical experimentation. Extensive efforts are being made to identify robust biomarkers to select patients who may benefit from treatment with ICIs. Tumor mutation burden (TMB) may be a relevant biomarker of response to ICIs in different tumor types; however, its clinical use is challenged by the analytical methods required for its evaluation. The possibility of using targeted next-generation sequencing panels has been investigated as an alternative to the standard whole exome sequencing approach. However, no standardization exists in terms of genes covered, types of mutations included in the estimation of TMB, bioinformatics pipelines for data analysis, and cut-offs used to discriminate samples with high, intermediate or low TMB. Bioinformatics serve a relevant role in the analysis of targeted sequencing data and its standardization is essential to deliver a reliable test in clinical practice. In the present study, cultured and formalin-fixed, paraffin-embedded cell lines were analyzed using a commercial panel for TMB testing; the results were compared with data from the literature and public databases, demonstrating a good correlation. Additionally, the correlation between high tumor mutation burden and microsatellite instability was confirmed. The bioinformatics analyses were conducted using two different pipelines to highlight the challenges associated with the development of an appropriate analytical workflow.

## Introduction

Immune-oncology therapeutics represent a novel approach to cancer therapy that has been demonstrated to significantly improve outcomes and quality of life of patients with different types of cancer. Several immune checkpoint inhibitors (ICIs) have already been approved for clinical practice or are in advanced phases of clinical experimentation; however, only a small proportion of patients achieve a long-lasting response to treatment with ICIs (1-3). Therefore, numerous efforts are being made to identify robust biomarkers to correctly select patients who may benefit from this therapy in order to decrease side effects and cost.

At present, the expression levels of PD-L1 and microsatellite instability (MSI) are the only predictive biomarkers approved for patient selection, but responses are also registered in certain patients with low PD-L1 expression or microsatellite stability (MSS) (4-6). Results from several studies have suggested that tumor mutation burden (TMB) may be an additional biomarker of response to ICIs in different tumor types (6-15).

TMB is defined as the total number of somatic mutations per coding area of a tumor genome. The rationale of its use as a biomarker is based on the fact that somatic mutations may lead to the formation of tumor-specific neoantigens, which are able to trigger T-cell activation against tumor cells (16-19). There is a large variability in mutation burden among and within tumor types, ranging from just a few to thousands of mutations (20-22). Results from several studies have demonstrated that subsets of patients with high TMB exist across almost all cancer types (12,14).

TMB and PD-L1 expression only partially overlap. Therefore, TMB may be complementary to PD-L1. Notably, the co-expression of the two biomarkers (high TMB/PD-L1 >50%) has previously been used to identify a subgroup of patients with the highest response rate (8,23). Similarly, patients with high TMB and high expression levels of genes associated with

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a T-cell inflamed state have been reported to exhibit the best outcome when treated with ICIs (24). Notably, TMB is also emerging as a prognostic marker that may aid in better stratifying patients with colon or lung cancer (25,26).

In this scenario, the possible use of TMB as a predictive and/or prognostic biomarker is challenged by the methods required for its evaluation. Whole-exome sequencing (WES) is the standard for TMB measurement, since it is able to identify all the possible somatic mutations in the entire tumor genome that may lead to the expression of neoantigens. However, this approach is clearly difficult to be translated into clinical practice, due to cost, amount of material required, complexity of output data and turnaround time. Therefore, the possible use of targeted next-generation sequencing (NGS) panels has been investigated as an alternative to WES. Panels that cover just a few hundred genes and at least ~1 megabase (Mb) of the human coding genome have been reported to correlate efficiently with TMB calculated by WES, in terms of TMB values and clinical responses observed in patients (11,27-30). Nevertheless, the panels used in these previous studies, as well as the assays that will enter the market in the future, may vary substantially in terms of genes covered, cut-offs used to discriminate samples with high or low TMB, and bioinformatics pipelines used for data analysis. Standardization and harmonization of these approaches are needed in order to provide a robust and reproducible biomarker.

Bioinformatics serve a relevant role in the analysis of targeted sequencing data and its standardization is essential in order to deliver a reliable test for use in clinical practice. In addition, the definition of TMB and, in particular, of the type of mutations (both synonymous and non-synonymous versus only non-synonymous) that should be included in the estimation of TMB have changed over the time. In this respect, the aim of the present study was to describe the results obtained with the first commercially available panel for TMB measurement, i.e. the Oncomine™ Tumor Mutational Load (OTML) assay, and the work undertaken to develop an appropriate bioinformatics analytical pipeline.

**Materials and methods**

*Samples and DNA extraction.* Cultured cell lines and formalin-fixed paraffin-embedded (FFPE) samples were obtained from two different sources. The following human tumor cell lines were obtained from American Type Culture Collection: Colorectal carcinoma Colo320, RKO, LoVo, HCT116, HT29, SW1116 and LS174T cell lines, and the lung carcinoma NCI-H1650 and NCI-H1975 cell lines. Cultured cell lines were maintained in a humidified atmosphere at 37°C and 5% $CO_2$. Colo320, NCI-H1650 and NCI-H1975 cells were grown in RPMI 1640 medium with GlutaMAX supplemented with 10% fetal bovine serum (FBS) (all from Thermo Fisher Scientific, Inc.). RKO and LS174T cells were cultured in Eagle's Minimum Essential Medium with GlutaMAX (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS. Lovo cells were maintained in F-12K medium with GlutaMAX (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS. HCT116 and HT29 cells were grown in McCoy's 5a medium (Gibco; Thermo Fisher Scientific, Inc.) modified plus GlutaMAX and 10% FBS. SW1116 cells were cultured in Leibovitz's L-15

medium with GlutaMAX (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS.

The following cell lines were provided as FFPE samples from AccuRef Diagnostics: Lung carcinoma A549 and H2228 cell lines, the colorectal carcinoma HCC2998 cell line, the melanoma SK-MEL-2 cell line, and the breast cancer MCF7 and T47D cell lines. Genomic DNA (gDNA) was isolated from the cultured cell lines using the DNeasy Blood & Tissue kit (Qiagen) and from FFPE cell lines using the RecoverAll Total Nucleic Acid Isolation kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturers' protocols. The gDNA was quantified using the dsDNA HS assay kit on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Inc.). The MSI status and POLE/POLD1 mutational status for the 15 cell lines, as derived from public data (31,32) are reported in Table I.

*OTML assay.* The OTML assay (Thermo Fisher Scientific, Inc.) is a targeted NGS panel that covers 409 genes with known cancer associations. Libraries were prepared using Ion AmpliSeq library kit plus (Thermo Fisher Scientific, Inc.) starting from 10 ng gDNA for each pool. Each library (50 pmol) was multiplexed and then clonally amplified by emulsion PCR and enriched on the Ion Chef instrument for automated template preparation using the Ion 540™ Chef kit, according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). Finally, the template was loaded on an Ion 540™ chip and sequenced on an Ion S5XL sequencer (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols (each sequenced chip contained eight samples).

*TMB data analysis.* The NGS results were analyzed using the default setting of two versions of the integrated pipelines, OTML v1.2 DNA Single Sample on Ion Reporter software v.5.6 and OTML v2.0 DNA Single Sample, available on the Ion Reporter software v.5.10 (Thermo Fisher Scientific, Inc.), which will be referred to as version A and B, respectively.

In both versions of the analysis workflow, the sequenced reads are aligned to human reference genome hg19 and the resulting BAM files are transferred to Ion Reporter software for variant calling. Several parameters of NGS analysis were used to consider a sample suitable for the inclusion in the comparison analysis with the two pipelines: Number of mapped reads, >5,000,000x; mean read depth, >300x; percentage of reads on target, >90%; target base coverage at 100x, >95%; uniformity, >80%. TMB calculation does not require matched normal samples. After the variant calling, a filter chain was applied to remove germline variants using population databases (1000 Genome Project, https://www.internationalgenome.org/data/; NHLBI GO Exome Sequencing Project https://esp.gs.washington.edu/drupal/node/1; and ExAC, https://bigd.big.ac.cn/databasecommons/database/id/3774), and to select somatic variants which have a minimum depth of base coverage above 60x to be used for TMB calculation without generating noise. In version A, TMB is calculated by counting synonymous and non-synonymous somatic single-base substitutions (SNVs), at or above 5% allelic frequency (AF), from the full panel, which is 1.2 Mb exonic and 0.45 Mb intronic. In version B, only the non-synonymous SNVs and short insertion-deletion mutations (InDels) with ≥5% AF are considered, derived from

Table I. List of cell lines.

| Cell line | MSI status/other relevant alteration | Tumor location, histology | Type of sample |
|---|---|---|---|
| Colo320 | MSS | Large intestine, carcinoma, adenocarcinoma | Cultured cells |
| H1650 | MSS | Lung carcinoma, non-small cell carcinoma | Cultured cells |
| H1975 | MSS | Lung, carcinoma, adenocarcinoma | Cultured cells |
| HT29 | MSS | Large intestine, carcinoma, NS | Cultured cells |
| SW1116 | MSS | Large intestine, carcinoma, adenocarcinoma | Cultured cells |
| HCT116 | MSI | Large intestine, carcinoma, NS | Cultured cells |
| LoVo | MSI/POLD1 | Large intestine, carcinoma, adenocarcinoma | Cultured cells |
| LS174T | MSI | Large intestine, carcinoma, adenocarcinoma | Cultured cells |
| RKO | MSI | Large intestine, carcinoma, NS | Cultured cells |
| A549 | MSS | Lung, carcinoma, NS | FFPE cells |
| H2228 | MSS | Lung, carcinoma, non-small cell carcinoma | FFPE cells |
| HCC2998 | MSS/POLE | Large intestine, carcinoma, adenocarcinoma | FFPE cells |
| MCF7 | MSS | Breast, carcinoma, NS | FFPE cells |
| T47D | MSS | Breast, carcinoma, ductal carcinoma | FFPE cells |
| SK-MEL-2 | MSI | Skin, malignant melanoma, NS | FFPE cells |

FFPE, formalin-fixed paraffin-embedded; MSI, microsatellite instability; MSS, microsatellite stability; NS, not specified.

the 1.2-Mb exonic region. The number of mutations counted is divided by the number of bases with sufficient coverage, to normalize the TMB values. The variant calling in version A is optimized only for the TMB calculation and not for variant detection, whereas the workflow in version B is also able to call clinically relevant variants in a specific predefined set of genes with a limit of detection (LOD) of 5% AF. Finally, following application of the filter chain, workflow B applies calibration to bring the TMB values closer to WES-based TMB. The calibration factor was calculated through linear modelling on The Cancer Genome Atlas database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). Workflow A does not apply calibration.

At the end of each analysis, a report was provided that included the normalized mutation load, defined as the number of somatic mutations per Mb of genome with an average coverage not less than 300x. In addition, the report summarized other information on the samples, including coverage, variants called, mutation signatures of the somatic variants, mutations consistent with deamination, UV and tobacco smoke damage.

*Statistical analysis.* Pearson's correlation test was used to evaluate the correlation between the mutation load/Mb obtained with workflow A and workflow B on 13/15 cell lines and the mutation count obtained with the massively parallel sequencing available on cBioPortal (https://www.cbioportal.org/). The correlation between TMB scores and the MSI status was assessed in 14 out of the 15 cell lines included in the present study. Mann-Whitney ranked sum test was used for subgroup comparisons (MSS vs. MSI groups). The Kruskal-Wallis test was used to compare the mean TMB value of the two workflows in all types of cell lines and in the selected MSS- and MSI-colorectal cell lines. Dunn's multiple comparison test was used as post-hoc analysis. All the statistical analyses were performed using the GraphPad PRISM version 5 for windows

(GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Targeted sequencing analysis with OTML assay on cultured and FFPE cell lines.* A total of 15 tumor cell lines, including nine cultured and six FFPE cell lines, were analyzed using the OTML assay. The panel included five MSI-high cell lines, nine MSS cell lines and one MSS/POLE mutated cell line (Table I), as defined according to the literature and public databases (31-33). The raw data underwent bioinformatics analysis with the integrated workflows. The obtained results were compared with each other and to the expected data from the literature and public databases (31-33).

To begin with, the obtained results on the entire cohort of cell lines were comprehensively analyzed using the bioinformatics workflows A and B, as aforementioned. The median TMB values were 15.87 (range, 4.98-179.2) and 8.38 (range, 2.56-176.5) with version A and B, respectively. The MSS cell line, HCC2998, which carries the POLE p.P286R (c.857C>G) mutation, had the highest TMB score among all the analyzed samples, with a TMB of 179.2 with workflow A and 176.5 with workflow B (Table II).

The bioinformatics pipeline is also able to identify mutations consistent with hydrolytic deamination of cytosine that generates uracil, a mechanism that is generally caused by formalin fixation. This phenomenon results in sequencing artifacts, which may cause an overestimation of TMB values. In particular, the workflows of analysis report as probable deamination mutations all the C:G>T:A variants detected below an AF of 15%. Since the parameters for variant calling are less stringent in workflow B, in order to improve sensitivity to call the variants, more deamination variants may be included in TMB calculation. In fact, when comparing

Table II. TMB results with version A and B of the Oncomine™ Tumor Mutation Load bioinformatics pipeline. Cell lines are listed from the highest TMB value to the lowest according to version A.

| Cell line | Mutation load per MB, version A | Mutation load per MB, version B | Estimated SNP proportion consistent with deamination, version A | Estimated SNP proportion consistent with deamination, version B | Mutation count[a] |
|---|---|---|---|---|---|
| HCC2998 | 179.17 | 176.5 | 0 | 3 | NA |
| RKO | 100.77 | 102.45 | 0 | 4 | 382 |
| LoVo | 77.61 | 74.27 | 1 | 13 | 267 |
| HCT116 | 74.62 | 63.35 | 0 | 2 | 227 |
| LS174T | 54.26 | 58.19 | 0 | 1 | NA |
| SK-MEL-2 | 33.83 | 18.72 | 0 | 4 | 90 |
| HT29 | 15.89 | 10.9 | 0 | 2 | 64 |
| SW1116 | 15.87 | 8.38 | 0 | 1 | 40 |
| H1975 | 10.37 | 6.69 | 0 | 1 | 54 |
| Colo320 | 9.78 | 5.88 | 0 | 1 | 23 |
| H2228 | 8.69 | 6.81 | 0 | 1 | 37 |
| H1650 | 6.74 | 5.04 | 0 | 2 | 25 |
| A549 | 6.25 | 7.57 | 0 | 1 | 34 |
| MCF7 | 6.16 | 3.39 | 0 | 0 | 19 |
| T47D | 4.98 | 2.56 | 0 | 2 | 21 |

The formalin-fixed paraffin-embedded cell lines are underlined. [a]Mutation count included nucleotide substitutions (synonymous and non-synonymous) and short insertion and deletion mutations (31). TMB, tumor mutation burden; SNP, single nucleotide polymorphism; NA, not applicable.

the results obtained with the two workflows, it was revealed that the number of variants compatible with deamination increased with version B, irrespective of the type of sample analyzed (Table II). In particular, the estimated number of possible deamination mutations ranged between 0 and 4 with version B, with the exception of the LoVo cell line, which had 13 mutations consistent with deamination. The LoVo cell line was also the only sample in which a deamination event was identified with version A. Notably, the TMB value in this specific sample was not affected by the presence of deamination mutations (Table II), possibly because workflow B considers only non-synonymous alterations, thereby including only a fraction of deamination mutations for TMB calculation.

*Correlation of TMB data.* In order to investigate the ability of this targeted panel to infer TMB, the TMB values were compared with the parallel sequencing data from >1,600 genes for the same cell lines available on cBioPortal and described in a study by Barretina *et al* (34). This analysis was performed on 13 out of 15 cell lines. The LS174T and HCC2998 cell lines were excluded, since both cell lines were not sequenced by Barretina *et al* (34). A significant correlation was observed between the results of the OTML assay and the mutations detected by wide genetic profiling (version A: P<0.0001 and r=0.9883; version B: P<0.0001 and r=0.9969; Fig. 1), with workflow B resulting in a stronger correlation. When the type of material used (FFPE vs. cultured) was taken into consideration, the correlation in FFPE samples was slightly lower than in cultured samples (data not shown). These data suggested that, despite the deamination mutation, the TMB may be robustly calculated using the OTML panel, both on cultured and FFPE

cell lines. Finally, the bioinformatics pipeline called all the genetic alterations reported in Cellosaurus for the HT-29 cell line (https://web.expasy.org/cellosaurus/CVCL_0320), apart from the PIK3CA mutation p.(Pro449Thr), which is not covered by the amplicons included in the panel (data not shown).

The TMB scores were compared with the MSI status, to observe whether an association was present between these two biomarkers, as expected. Since a mechanism of mutation accumulation independent from the MSI status is known to be present in the HCC2998 cell line (i.e. a POLE mutation), this sample was excluded from the comparisons. The median TMB value was revealed to be significantly increased in MSI-versus MSS-cell lines with the two workflows (Mann Whitney test, P=0.0010; Fig. 2), confirming an association between high mutation burden and MSI. The mean values of the MSS and MSI groups of the two workflows were comprehensively compared, revealing a significant difference in the observed means (Kruskal-Wallis test, P=0.0002) (data not shown). Post-hoc analysis with Dunn's multiple comparison test highlighted a significant association (P<0.05) in the following subgroups: MSS vs. MSI-H in both versions A and B; MSS v.B vs. MSI-H v.A was also significantly different (data not shown). Additionally, a significant difference was observed when the TMB values in the MSS- and MSI-colorectal cell lines only were compared (13.85 vs. 76.82 version A, P=0.0027; 8.39 vs. 74.56 version B, P=0.0024) (data not shown).

**Discussion**

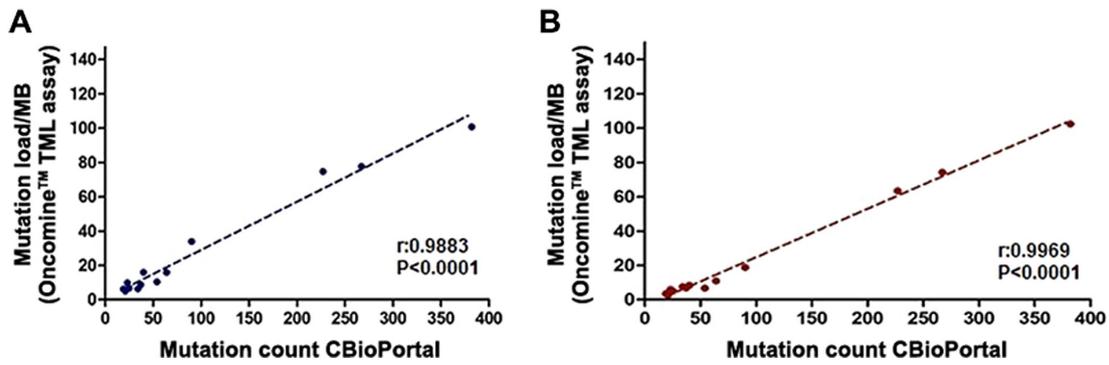Although TMB is emerging as a relevant biomarker for ICI treatment in different tumor types, the optimal method for

Figure 1. Correlation between Oncomine™ TML results and mutation count from parallel sequencing data of >1,600 genes. (A) Correlation between workflow A and mutation count; (B) correlation between workflow B and mutation count. TML, Tumor Mutational Load; MB, megabase.
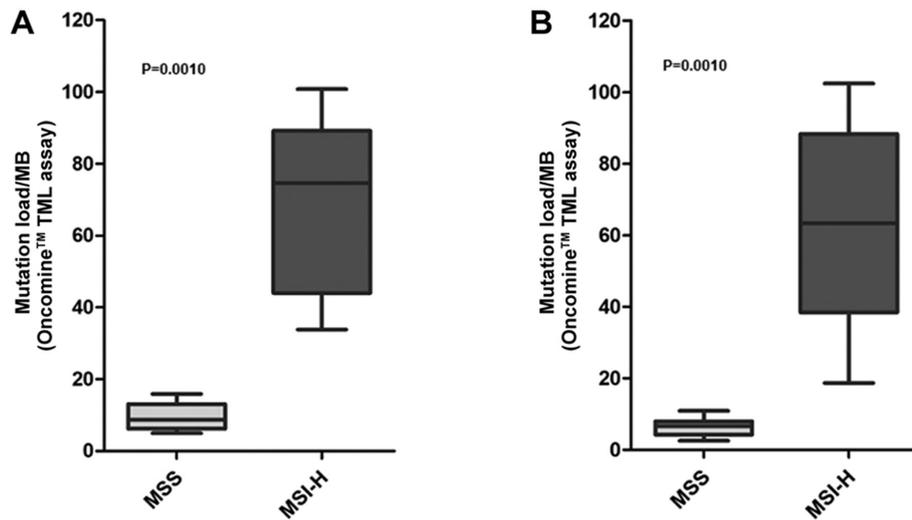


Figure 2. Comparison of TMB values according to MSI status. (A) Association of TMB, as calculated by workflow A, with MSI status; (B) association of TMB, as calculated by workflow B, with MSI status. TMB, tumor mutation burden; MSI, microsatellite instability-high; MSS, microsatellite stability; MB, megabase.

its calculation remains to be established. The use of targeted panels has demonstrated its efficacy in TMB measurement within clinical trials (13,23). However, the use of panels poses different considerations, starting from the coverage of the exome that should be ≥1 Mb (27), to the bioinformatics pipelines that are used to select the alterations to be counted for the TMB estimation (19,35). There is no consensus on the most appropriate approach for its calculation since the definitions of TMB are multiple and based on different assumptions (19,35). This lack of harmonization leads to TMB values that change depending on the panels used and thus are difficult to compare.

The panel used within the present study covers 1.7 Mb of genomic area and 1.2 Mb of exome area, and it has been demonstrated to be sufficient to estimate TMB accurately (36,37). However, within the same panel, adjustments to the analysis workflow have been done since its launch on the market and some of them substantially changed the way TMB is measured. Version A included only single-base substitutions, considering both synonymous and non-synonymous variants. Version B, despite including only non-synonymous alterations, also counts short InDels together with SNVs. The latter workflow is based on the correct assumption that only non-synonymous alterations may lead to the expression of neoantigens, which are responsible

for the antitumor immune response. However, the presence of random alterations is anyway suggestive of a tumor that may accumulate a high number of mutations, thus having a higher probability of presenting neoantigens. As the genomic region from which the TMB value is inferred is generally limited when using gene panels, the inclusion of non-coding regions and non-synonymous alterations may aid in the estimate of the TMB. Another aspect to consider is the different impact of SNVs and InDels on the potential generation of immunogenic antigens. Indeed, InDels alterations that cause a frameshift may produce neoantigenic peptides highly different from self, leading to a higher activation of T cells and an increased immune response (38,39). In this regard, the presence of frameshift InDels was significantly associated with response to checkpoint inhibitors in datasets of patients with melanoma (38). In another recent paper, an increased progression-free survival was associated with the presence of frameshift InDel burden in patients treated with ICIs (40). In addition, significantly different overall response rates and disease control rates were observed between patients with and without frameshift InDels (40). However, the data available on the role of InDels and the different possible impact on response to ICI are limited and further evaluation is required. At present, the identification of the most appropriate

TMB calculation remains controversial and thus opened to different interpretations.

Another issue is associated with the material used. FFPE-derived DNA may be difficult to use within NGS approaches due to possible artifacts. Analysis pipelines should take into consideration this problem and should provide information on the quality of the DNA tested. The Ion Reporter analysis workflow provides an estimation of the DNA quality, reporting the number of possible deamination artifacts. In the event of a case with a high number of artifacts, the data analysis with a LOD set at 10% could be able to cut out the variants consistent with deamination, which generally are present below 10-15% AF. This workflow, however, may cause the loss of information on TMB, since certain alterations that are present below 10% AF may be excluded from the TMB calculation.

In the present study, the TMB panel used was able to infer the TMB values as deducted from massive parallel sequencing analyses of a higher number of genes compared with the OTML panel (>1,600 vs. 409 genes), independently from the type of material used, thus suggesting that it is a robust method for TMB evaluation. However, there are certain limitations to the present study. For example, it is focused on a small cohort of cell lines and thus it is based on the use of artificial samples. On the other hand, the use of standardized samples has several advantages when describing the differences between two different bioinformatics pipelines. In particular, the use of a well-characterized set of samples, including cell lines, allows the comparison of these results with data from literature and public databases, and may be used to set the most suitable analysis approach to be transferred later in clinical samples. Therefore, the present study is a preliminary evaluation of the challenges and difficulties in harmonizing TMB data. In addition, to the best of our knowledge, the present study was the first to address the influence of the different bioinformatics pipelines on the TMB value. Given the lack of standardization of TMB testing and the recent FDA approval of TMB as a diagnostic biomarker for solid tumors, the results of the present study may be of high interest for the scientific community. However, further analyses are required to collect information on FFPE samples from patients, and also to retrieve clinical data from patients treated with immunotherapy, to confirm the consistency of the results.

In conclusion, the present data suggest that the two bioinformatics pipelines used in the present study for data analysis are able to correctly infer the TMB. Nevertheless, version B exhibited a slightly better correlation with TMB assessed by wide genomic profiling and it reflects the current definition of TMB, which includes only non-synonymous variants. As the present study was limited to cell lines, additional validation of the bioinformatics pipelines in clinical samples is required.

## Acknowledgements

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

NN designed the study. FF, RP and CR performed the experiments. FF, RP, REA and CR validated the results of the study. FF, RP, REA, FB, ML and CR performed formal analysis. FB, FF and RP collected and organized the data. FF and RC prepared the original draft. NN reviewed and edited the manuscript. CA, RC and FH verified the data and supported data analysis. NN supervised the study. NN and FF acquired the funding. All authors read and approved the final manuscript. FF and RP confirmed the authenticity of all the raw data.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

NN has received speaker's fee and is on the advisory boards of the following companies: Astra-Zeneca, Bayer, Illumina, Incyte, MSD, Merck KgA, J&J, Qiagen, Roche, Thermo Fisher Scientific, Inc. CA, FH and RC are Thermo Fisher Scientific, Inc. employees. Thermo Fisher Scientific, Inc. is the supplier of the Oncomine Tumor Mutation Load assay used within the study. The other authors declare no competing interests.

## References

1. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, Hassel JC, Rutkowski P, McNeil C, Kalinka-Warzocha E, *et al*: Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med 372: 320-330, 2015.
2. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, Patnaik A, Aggarwal C, Gubens M, Horn L, *et al*; KEYNOTE-001 investigators: Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med 372: 2018-2028, 2015.
3. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WE, Poddubskaya E, Antonia S, Pluzanski A, Vokes EE, Holgado E, *et al*: Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. N Engl J Med 373: 123-135, 2015.
4. Patel SP and Kurzrock R: Pd-l1 expression as a predictive biomarker in cancer immunotherapy. Mol Cancer Ther 14: 847-856, 2015.
5. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, Gottfried M, Peled N, Tafreshi A, Cuffe S, *et al*; KEYNOTE-024 Investigators: Pembrolizumab versus chemotherapy for pd-l1-positive non-small-cell lung cancer. N Engl J Med 375: 1823-1833, 2016.
6. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, *et al*: Pd-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 372: 2509-2520, 2015.
7. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, *et al*: Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med 371: 2189-2199, 2014.

8. Carbone DP, Reck M, Paz-Ares L, Creelan B, Horn L, Steins M, Felip E, van den Heuvel MM, Ciuleanu TE, Badin F, et al; CheckMate 026 investigators: First-line nivolumab in stage iv or recurrent non-small-cell lung cancer. N Engl J Med 376: 2415-2426, 2017.
9. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, Sucker A, Hillen U, Foppen MHG, Goldinger SM, et al: Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science 350: 207-211, 2015.
10. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, Yuan J, Wong P, Ho TS, et al: Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 348: 124-128, 2015.
11. Rizvi H, Sanchez-Vega F, La K, Chatila W, Jonsson P, Halpenny D, Plodkowski A, Long N, Sauter JL, Rekhtman N, et al: Molecular determinants of response to anti-programmed cell death (pd)-1 and anti-programmed death-ligand 1 (pd-l1) blockade in patients with non-small-cell lung cancer profiled with targeted next-generation sequencing. J Clin Oncol 36: 633-641, 2018.
12. Samstein RM, Lee CH, Shoushtari AN, Hellmann MD, Shen R, Janjigian YY, Barron DA, Zehir A, Jordan EJ, Omuro A, et al: Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nat Genet 51: 202-206, 2019.
13. Hellmann MD, Ciuleanu TE, Pluzanski A, Lee JS, Otterson GA, Audigier-Valette C, Minenza E, Linardou H, Burgers S, Salman P, et al: Nivolumab plus ipilimumab in lung cancer with a high tumor mutational burden. N Engl J Med 378: 2093-2104, 2018.
14. Yarchoan M, Hopkins A and Jaffee EM: Tumor mutational burden and response rate to pd-1 inhibition. N Engl J Med 377: 2500-2501, 2017.
15. Rosenberg JE, Hoffman-Censits J, Powles T, van der Heijden MS, Balar AV, Necchi A, Dawson N, O'Donnell PH, Balmanoukian A, Loriot Y, 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 387: 1909-1920, 2016.
16. Brown SD, Warren RL, Gibb EA, Martin SD, Spinelli JJ, Nelson BH and Holt RA: Neo-antigens predicted by tumor genome meta-analysis correlate with increased patient survival. Genome Res 24: 743-750, 2014.
17. Schumacher TN and Schreiber RD: Neoantigens in cancer immunotherapy. Science 348: 69-74, 2015.
18. Anagnostou V, Smith KN, Forde PM, Niknafs N, Bhattacharya R, White J, Zhang T, Adleff V, Phallen J, Wali N, et al: Evolution of neoantigen landscape during immune checkpoint blockade in non-small cell lung cancer. Cancer Discov 7: 264-276, 2017.
19. Büttner R, Longshore JW, López-Ríos F, Merkelbach-Bruse S, Normanno N, Rouleau E and Penault-Llorca F: Implementing TMB measurement in clinical practice: Considerations on assay requirements. ESMO Open 4: e000442, 2019.
20. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Børresen-Dale AL, et al; Australian Pancreatic Cancer Genome Initiative; ICGC Breast Cancer Consortium; ICGC MMML-Seq Consortium; ICGC PedBrain: Signatures of mutational processes in human cancer. Nature 500: 415-421, 2013.
21. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, Carter SL, Stewart C, Mermel CH, Roberts SA, et al: Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 499: 214-218, 2013.
22. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr and Kinzler KW: Cancer genome landscapes. Science 339: 1546-1558, 2013.
23. Ready N, Hellmann MD, Awad MM, Otterson GA, Gutierrez M, Gainor JF, Borghaei H, Jolivet J, Horn L, Mates M, et al: First-line nivolumab plus ipilimumab in advanced non-small-cell lung cancer (checkmate 568): Outcomes by programmed death ligand 1 and tumor mutational burden as biomarkers. J Clin Oncol 37: 992-1000, 2019.
24. Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, Sher X, Liu XQ, Lu H, Nebozhyn M, et al: Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. Science 362: 362, 2018.
25. Innocenti F, Ou FS, Qu X, Zemla TJ, Niedzwiecki D, Tam R, Mahajan S, Goldberg RM, Bertagnolli MM, Blanke CD, et al: Mutational analysis of patients with colorectal cancer in calgb/swog 80405 identifies new roles of microsatellite instability and tumor mutational burden for patient outcome. J Clin Oncol 37: 1217-1227, 2019.
26. Owada-Ozaki Y, Muto S, Takagi H, Inoue T, Watanabe Y, Fukuhara M, Yamaura T, Okabe N, Matsumura Y, Hasegawa T, et al: Prognostic impact of tumor mutation burden in patients with completely resected non-small cell lung cancer: Brief report. J Thorac Oncol 13: 1217-1221, 2018.
27. Chalmers ZR, Connelly CF, Fabrizio D, Gay L, Ali SM, Ennis R, Schrock A, Campbell B, Shlien A, Chmielecki J, et al: Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med 9: 34, 2017.
28. Frampton GM, Fichtenholtz A, Otto GA, Wang K, Downing SR, He J, Schnall-Levin M, White J, Sanford EM, An P, et al: Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. Nat Biotechnol 31: 1023-1031, 2013.
29. Roszik J, Haydu LE, Hess KR, Oba J, Joon AY, Siroy AE, Karpinets TV, Stingo FC, Baladandayuthapani V, Tetzlaff MT, et al: Novel algorithmic approach predicts tumor mutation load and correlates with immunotherapy clinical outcomes using a defined gene mutation set. BMC Med 14: 168, 2016.
30. Campesato LF, Barroso-Sousa R, Jimenez L, Correa BR, Sabbaga J, Hoff PM, Reis LF, Galante PA and Camargo AA: Comprehensive cancer-gene panels can be used to estimate mutational load and predict clinical benefit to PD-1 blockade in clinical practice. Oncotarget 6: 34221-34227, 2015.
31. Berg KCG, Eide PW, Eilertsen IA, Johannessen B, Bruun J, Danielsen SA, Bjørnslett M, Meza-Zepeda LA, Eknaes M, Lind GE, et al: Multi-omics of 34 colorectal cancer cell lines - a resource for biomedical studies. Mol Cancer 16: 116, 2017.
32. Catalogue Of Somatic Mutations In Cancer (COSMI): Cell Lines Project v92, released 27-AUG-20. Available from: https://cancer.sanger.ac.uk/cell_lines. Accessed August 28, 2019.
33. Gupta A, Connelly C, Frampton G, Chmielecki J, Ali S, Suh J, Schrock A, Ross J, Stephens P and Miller V: The druggable mutation landscape of lung adenocarcinoma. J Thorac Oncol 12: 977, 2017.
34. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehár J, Kryukov GV, Sonkin D, et al: The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 483: 603-607, 2012.
35. Fenizia F, Pasquale R, Roma C, Bergantino F, Iannaccone A and Normanno N: Measuring tumor mutation burden in non-small cell lung cancer: Tissue versus liquid biopsy. Transl Lung Cancer Res 7: 668-677, 2018.
36. Chaudhary R, Quagliata L, Martin JP, Alborelli I, Cyanam D, Mittal V, Tom W, Au-Young J, Sadis S and Hyland F: A scalable solution for tumor mutational burden from formalin-fixed, paraffin-embedded samples using the Oncomine Tumor Mutation Load Assay. Transl Lung Cancer Res 7: 616-630, 2018.
37. Endris V, Buchhalter I, Allgäuer M, Rempel E, Lier A, Volckmar AL, Kirchner M, von Winterfeld M, Leichsenring J, Neumann O, et al: Measurement of tumor mutational burden (TMB) in routine molecular diagnostics: In silico and real-life analysis of three larger gene panels. Int J Cancer 144: 2303-2312, 2019.
38. Turajlic S, Litchfield K, Xu H, Rosenthal R, McGranahan N, Reading JL, Wong YNS, Rowan A, Kanu N, Al Bakir M, et al: Insertion-and-deletion-derived tumour-specific neoantigens and the immunogenic phenotype: A pan-cancer analysis. Lancet Oncol 18: 1009-1021, 2017.
39. Mandal R, Samstein RM, Lee KW, Havel JJ, Wang H, Krishna C, Sabio EY, Makarov V, Kuo F, Blecua P, et al: Genetic diversity of tumors with mismatch repair deficiency influences anti-PD-1 immunotherapy response. Science 364: 485-491, 2019.
40. Chae YK, Viveiros P, Lopes G, Sukhadia B, Sheikh MM, Saravia D, Florou V, Sokol ES, Frampton GM, Chalmers ZR, et al: Clinical and immunological implications of frameshift mutations in lung cancer. J Thorac Oncol 14: 1807-1817, 2019.