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The presented manuscript uses a large dataset with 970 NSCLC samples to illustrate that TMB in NSCLC samples is comparable between treatment-naïve and treatment-experienced patients. Similar trends have been previously shown, but never in as large a dataset as here. We believe the article adds valuable insights in this field that may have a direct impact on how TMB is used for patient stratification.

That being said, with such a large set of comprehensively profiled tumors we would hope to see a bit content to the article. In particular, in-depth analysis of specific mutations present in these samples (i.e. predictive for IO-therapy, targeted therapies, etc) would be desirable.

Please see our point-by-point review for suggestions to improve the manuscript as well as requests to address some ambiguities that we believe should be addressed prior to publication.

Tumor mutational burden (TMB), an indirect measure of tumor-derived neoantigens, has emerged as a promising biomarker for ICI patient stratification. Different platforms interrogating TMB exist and clear guidelines are missing. Retrospective studies are important to address the impact of pre-analytical variables in heterogenous patient cohorts and help to assess clinical utility. Since ICIs are often used in advanced stages, patients frequently undergo multiple treatment cycles before receiving ICI therapy.

The authors of this article address how previous treatment impacts TMB, exploring the difference in TMB between "treatment- experienced" (n=155) and "treatment- naive" patients (n=815). In particular, the authors retrospectively combined next-generation sequencing data with clinical data and assessed the effect of chemotherapy and radiation therapy on TMB status. DNA mutations were assessed using a custom SureSelect XT assay covering 592 genes. TMB was estimated by counting the coding variants, excluding synonymous and germline mutations.

The authors report no statistically significant differences in TMB between treatment-naïve and treatment-experienced patients, in line with what has been previously reported by Sakai et al. (DOI:https://doi.org/10.1016/j.lungcan.2018.11.025), but with a much more robust sample cohort. From a diagnostic perspective, these findings are important for patient stratification since they show that pre- and post-treatment samples are comparable in their TMB distribution, undermining the need to implement different cut-offs for TMB-high according to treatment history.

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General remarks:

The language and style of writing are appropriate and understandable. The tables are clear and readable. The figures are properly labelled and easy to read.

Specific Comments

Thank you for your feedback. Please see responses below. The line numbers refer to the document with the track changes. Thank you for your consideration of our manuscript for publication.

Major Points:

Comment 1

The authors have used the SureSelect XT assay by Claris including 592 whole gene targets. TMB has been estimated by calculation of all coding variants, except synonymous and germline mutations. We assume the authors also investigated the presence of genetic variants in other genes, than just the oncogenic drivers. Given the great amount of data (n=970), a mutational profiling including statistical analysis would be welcome and could provide more insight about specific mutations in treatment-naive or treatment- experienced patients (e.g. in the form of an Oncoprint, Figure 2 in https://doi.org/10.1016/j.ccell.2018.03.018 & Figure 4 in doi:10.1002/path.5344). We believe that the addition and discussion (i.e. presence of known resistance mutations for IO therapy) of this data in an additional figure would greatly enhance the value of the article.

Reply 1:

We agree that this analysis would be insightful and add to the value of this manuscript. However, after discussion with representatives from Caris, the addition of this information is not feasible at this time.

Comment 2

Formalin fixation causes deamination artefacts that can later influence the mutational profiling (C:G>T:A variant artifacts) and lead to potential overestimation of TMB. Was the potential presence of FFPE artifacts considered in the variant detection and verification pipeline? Were samples pre-treated to compensate for potential FFPE artefacts (i.e. UDG)?

Reply 2:

Caris is a CAP-CLIA certified lab and the TMB calculations are reported as part of a validated LDT. Appropriate measures are taken to account for any biases which may be incurred during tissue pre-processing and are addressed in their calling algorithm pipeline. Thus, any bias that

would impact TMB as a factor of FFPE treatment is minimized.

Comment 3

The authors stated that treatment- naive and - experienced groups were compared, at the same time it has been stated that the -naive group consisted primarily of primary tumor and the -experienced group of metastatic samples (line 152-154). Please add a figure or table comparing TMB in primary and metastatic groups according to treatment status. Figure 2 only shows the difference between different smoking status but leaves out the information about the treatment status. The proportion of metastatic cases is higher in the treatment-experienced group. Since higher TMB is observed in metastatic cases why is the TMB not significantly higher in the treatment- experienced group? Can the authors further comment on this?

Reply 3:

We have added this information in Table 3 and in the text (line 176-181). Although TMB is higher in metastatic tissue sources, the overall difference is small. When accounting for other factors that may contribute to TMB, our study did not find a statistically significant difference in TMB between the treatment-naïve and treatment-experienced cohorts.

Comment 4

The authors show a significant difference in TMB between (former) smokers and never smokers, as well as between oncogenic-driven and wild-type patients. Are the never smokers the same patients as the oncogenic-driven patients since both groups show a median TMB of 4 mut/mb? This comparison is missing and should be added and discussed.

Reply 4:

The oncogene-mutated patients included never smokers, former smokers, and current smokers. The fact that the median TMB was the same is coincidental. Details are added in lines 213-215

Minor Points:

Comment 5

Line 70: The sentence “even after accounting for all coding variants in TMB analysis” is confusing. Is this referring to the comparison between oncogenic-driven and oncogenic wild-type samples? Please elaborate and remove or rephrase if necessary.

Reply 5: This sentence was in reference to the method used to calculate TMB (had been discussed in the methods). However, we agree that it is confusing and we have removed it from the manuscript.

Comment 6

Line 98: It is not clear form the text if all samples had TCC of 20% or if this was the minimum TCC. Please rephrase.

Reply 6:

20% TCC is the minimum requirement. This was updated (line 97)

Comment 7

The authors mention an "initial algorithm" for calculating TMB (line 113) but then never use this algorithm. According to line 162 not, thus it is useless to describe it in the methods. This also applied to lines 220 – 230, where the authors discuss the differences between the old and the new algorithm. Since no data from the old algorithm is shown in the study, we don't believe it adds any value to the article to include this discussion. We suggest removing this part.

Reply 7:

We agree that the comparison of initial and updated algorithm adds little value and is confusing. We have removed mentions of the two algorithms in the methods, results, and discussion sections.

Comment 8

The authors often use "patient" and "sample" interchangeably and it is not clear if each sample corresponded to an individual patient or if there were patients with multiple samples in the study. This should be clarified in the methods or results part.

Reply 8:

Tumor specimens in which the same block was previously tested (n=8) were excluded (line 139-140). To clarify as above, we added this more explicitly in the text (line 139-141) Also, a flow chart was added (Figure 1).

Comment 9

Table 1 is never mentioned in the text (Table 1 and Line 145-159)

Reply 9:

Please see line 152 which mentions table 1.

Comment 10

Information on tumor staging is not given in Table 1. It has been recently shown that patients

with early stage NSCLC have a higher TMB in comparison to advanced stages and more often show a SBS4 signature, which is associated with tobacco smoking (see https://www.esmo.org/oncology-news/patients-with-early-stage-nsclc-have-a-higher-tmb-in-comparison-to-advanced-stage-disease). If staging information is available it could be interesting to investigate if the authors see the same in their cohort.

Reply 10:
We agree that the addition of stage would be an interesting analysis. However, staging information was not collected in the data points of the initial study and cannot be retrospectively collected at this time.

Comment 11
Line 147: "885 patients were radiation-naïve and 842 patients were chemotherapy-naïve". This phrasing is a bit confusing. We would prefer to see how many of the 155 patients where treated with chemo or radiation.

Reply 11:
This is revised in the text (Line 151).

Comment 12
A flow chart would help to make the reader understand how the groups are distributed since several numbers are mentioned.

Reply 12:
A flow chart was added. Please see figure 1.

Comment 13
In general, it would be very helpful to provide 95% CI next to median, eventually also the range of sample distribution.

Reply 13:
Calculated CI for the median values have been added throughout the manuscript. Please note that the CI for medians are generated from a pseudomedian. The pseudomedian estimate is based on the F distribution and is equal to the median when data are symmetric. The CI interval on the pseudomedian coincides with the Wilcox test p-value.

Comment 14
A previous report from Sakai et al. (DOI:https://doi.org/10.1016/j.lungcan.2018.11.025)

similarly addresses the impact of therapy on TMB. In particular, Sakai assesses TMB in matched pre- and post-treatment specimens. While finding no clear trend, Sakai et al. show that TMB changes dramatically in some of the matched specimens. The authors should comment on this, including this reference in their discussion.

Reply 14

Thank you for providing this important reference. This data is added to the discussion (line 282-285)

Figure Legends:

Comment 15

Individual p values for each comparison is needed to understand differences in subgroups

Reply 15

Individual p values were added to compare between smoking subgroups in the figure legend.

Comment 16

Figure 1&2: Separation across smoking status is not mentioned in the legend

Reply 16

The smoking status designation is written below the figure. Individual p values were added to compare between smoking subgroups in the figure legend.

Comment 17

Line 232: “As expected, TMB....” Please add reference confirming this statement Line 236: “TMB is negatively correlated with clinical outcomes of EGFR-mutated NSCLC....” Please add reference Line 242: “change in TMB would be detectable in the same individual before and after chemotherapy.....” Please add and discuss Sakai et al. (DOI:https://doi.org/10.1016/j.lungcan.2018.11.025)

Reply 17:

The references were added as indicated. The reference and discussion of the manuscript by Sakai and collogues was also added to the discussion (line 282-285).