Ref.: **EGFR AND KRAS MUTATIONS IN THE NON-TUMORAL LUNG. PROGNOSIS IN PATIENTS WITH ADENOCARCINOMA.**
jcm-469642

Dear Reviewer #2 and Editor,

In the text attached below, please find the answer to your comments about the manuscript submitted with the reference jcm-469642.

Sincerely yours,

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Point by Point responses to Reviewers

Reviewer #2

1. *Very interesting study. However, the results are superficially presented and need significant improvement. Non-neoplastic tissues are removed from tissues adjacent to ADCs. According to the details information provided by the author, a minimum margin of 50px is considered. One major limitation of this procedure is the increased chance for sample cross contamination during surgical procedures. This needs to be clearly explained in the methodology section.*

In all of the patients included in our study, we used samples defined as "non-tumoral lung", always when the next two conditions were present: 1. All cases should have tumor-free resection margins assessed by an expert pathologist specialized in lung cancer (Dr. Lara Pijuan); and 2. After this histological diagnosis, the pathologist chose the area of lung farthest from the tumor (the distance to the tumor was never ever less than 2 cm) and the absence of tumor micro-invasion was confirmed, at least histologically, using the traditional methods required for diagnosis of malignancy.

Even though the presence of some changes in the 'normal cells' is an essential part of our hypothesis, it also implies that their morphological characteristics were still within normal limits. Therefore, we believe that an expert pathologist analysis using the standard histopathological methods widely employed in clinical practice actually allows us to conclude that all the samples were 'free of tumor invasion'. Nevertheless, we have previously performed a second-expert readying of all the non-tumoral samples by an additional lung pathologist. This analysis also confirmed the absence of invasion in the 'non-tumoral tissue'.

We can also confirm that in no case was vascular or lymphatic tumor invasion found at the time of diagnosis. The presence of circulating-tumor cells from blood vessels as an alternative explanation of our results is certainly possible. However, we think it is unlikely because of the following reasons: the presence of circulating tumoral DNA (ctDNA) and circulating tumoral cells strongly depends on the tumor size, metastasis status and TNM stage (Bettegowda C. et al, Sci Transl Med 2014:224; Ke-Zhong Chen et al, Sci Rep 2016;6), the specific detection of EGFR mutations in the blood of patients with early-stage lung cancer (stages I-II) is relatively low and the detection of KRAS by CastPCR in this population has not yet been reported although it is assumed to be very low (Pérez C et al, Transl Lung Cancer Res 2016:5). Since our cohort was composed of patients with early-stage lung cancer, with a presumably curative surgery, surgery margins were always negative, no vessel/lymphatic invasion was detected, and the quantity of blood cells in the sample of non-tumoral lung was probably very low, we can reasonably assume that the mutated DNA we identified did not come from circulating tumoral cells.

See changes in the new version of the manuscript (pag 3, lines 106-107; and pag 4, lines 142-143). If considered by the reviewers, a schematic approach of

the methodology could be added to the final manuscript and the results could be further deepened to clarify the pertinent doubts.

2. *Digital PCR was performed to identify point mutations in all samples. No quantitative data was provided from neither the ADC nor the neoplastic samples. This would be highly informative and strengthen the results. Also, the data could be compared with IHC results to assess whether the 2 experiments are consistent.*

To identify the driver mutations in non-tumoral tissue we performed a TaqMan Mutation Detection Assay that is an ultra-sensitive next-generation PCR, and has a minimum of 0.1% sensitivity since it can detect fewer than 10 copies of mutant DNA sequences. Although this technique theoretically allows us not only to detect but also to quantify copies of mutated genes, it is not the best method to perform a quantification of mutations copies. This is why we have performed an additional and different PCR technique, both for this reason and also to confirm the presence of driver mutations in the 10 samples of non-tumoral lung tissue from patients with detected mutations. The Digital PCR performed in the non-tumoral tissue demonstrated that in the cases where *EGFR* mutations were detected, a mean of 0.20% of mutated copies with respect to total copies of the gene were identified. Furthermore, we detected a minimum of 0.02% and a maximum of 0.50% of such copies.

In the cases where KRAS was detected, a mean of 0.08% of mutated copies with respect to total copies of the gene were identified, and we detected a minimum of 0.02% and a maximum of 0.17% of such copies.

These findings are included in the original manuscript (see pag 7, lines 172-175). However, to clarify this point see changes in the new version of the manuscript (pag 3, lines 115-116).

We thank the reviewer regarding the specific recommendation about making a comparison with IHC to assess whether the experiments are consistent. However, we have chosen an alternative method. The detection of *EGFR* and *KRAS* by immunohistochemistry is commonly considered as a valid approach. However, despite having a good specificity (from 67% to 98%), we have not considered it appropriate enough to validate the results of our study due to its limited sensitivity in comparison with PCR-based techniques (Hogman P et al, Annals of Oncology 2012; El-Zammar OA et al, Diag Mol Pathol 2009; Hitij NT et al, Clin Lung Cancer 2017; and Piton N et al G Research and Practice 2015). As we have analyzed non-tumoral tissue with a high prevalence of emphysema and we were expecting low mutation levels, we have chosen ultra-sensitive PCR approaches such as CastPCR (TaqMan based) and Digital PCR rather than the IHC assessment.

3. *The presence of mutations in non-tumoral tissues implies 2 phenomena: (i) local dissemination of cancer cells from parental tumors, in which case both parental and normal-like tissues should have a least one mutation in common; (ii) independent cells with driver mutations in which case the mutations may be different between the tumor and adjacent tissues. A pathological approach may not be the best strategy to evaluate the above*

possibilities. With regards to that, no strong evidence indicating non-tumoral tissues as the source of EGFR and KRAS mutations was provided. More direct evidence is needed (ei: Mutant Kras specific staining or FISH could be included with good negative controls).

We completely agree with the reviewer about the two possible phenomena that could explain the results. We are convinced, and this is a part of the main hypothesis of our study, that we are facing the second option because of the explanation we have mentioned before in our response to the first comment of Reviewer #2. There is also very recent and strong evidence in the line of our results in other tissues: E.g the results published by Nikolaev S et al. in the NEJM (2018, DOI: 10.1056/NEJMoa1709449), where the authors identified activating *KRAS* mutations in non-malignant tissue samples of patients with arteriovenous malformations of the brain, and by Anglesio M et al. who also published a paper in the NEJM (DOI: 10.1056/NEJMoa1614814) where they described for the first time the presence of driver-mutations in non-tumoral tissue of patients with endometriosis.

Regarding the specific comment about using *KRAS* specific staining or FISH for more direct evidence, we used more sensitive and specific tests in our study (CastPCR [TaqMan based Real-Time PCR] and Digital PCR) to confirm an quantify the mutations. These tests have a better performance in comparison with other techniques, especially in samples where a low mutational burden would be expected (as occurred in our study). However, if considered necessary by the reviewers and editor, a supplementary document can be added to delve into the more technical aspects of the molecular biology tests used in the study.

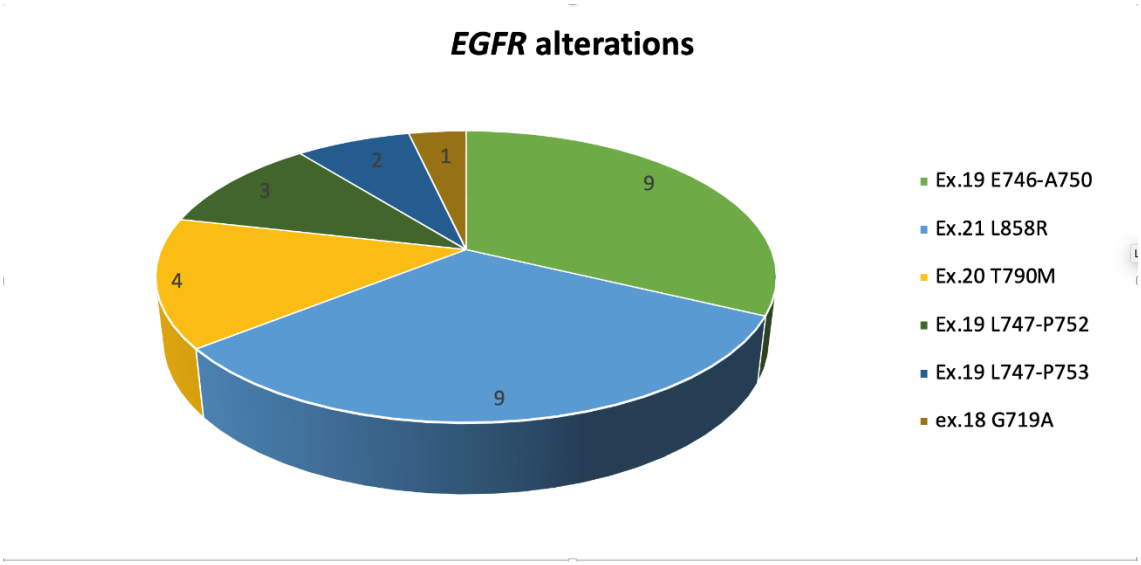
4. *Abbreviations such as SM are used throughout the manuscript without being clearly defined in the text.*

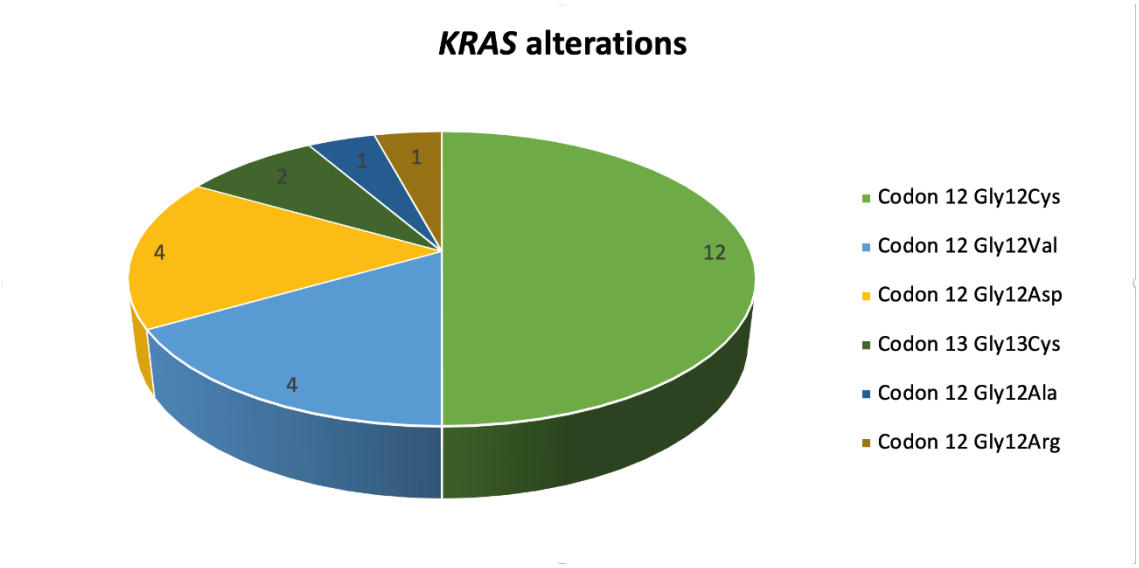
We thank the reviewer for his/her appreciations. Patients included in the ‘same mutation group’ (SM) are those with the same driver-mutation in the lung adenocarcinoma and the non-tumoral tissue. We have added a clear definition of this group in the new version of the manuscript (see pag 3, lines 126-130). Moreover, we can also add new paragraphs to improve definitions of other abbreviations if considered by the reviewers.

5. *Considering that a few patients have multiple mutations, it is important to evaluate how this could affect the recurrence or patient DFS rate. It is unclear whether the multiple mutations were identified in ADC or normal-like adjacent tissues. To assess possible connections between original tumor and adjacent tissues, a side-by-side comparison of identified mutations would be informative. Similarly, a graphical representation of EGFR/ KRAS exons and their mutation rate (table 2) would be appreciated by readers.*

The main objective of our study was to identify if patients with lung adenocarcinoma and *EGFR/KRAS* mutations could also have the same

molecular alterations in the histologically non-tumoral lung tissue (NTL). The results of our study are in line with our hypothesis, and additionally suggest a worse medium-term prognosis in those patients that presented the same mutation (SM group) in the tumor and the non-tumoral tissue when compared with the patients with mutations only in the former. The presence of multiple mutations only occurred in four patients with two different *EGFR* alterations (17.4% of patients with non-wild-type *EGFR* in the ADK). This phenomenon is called “comutations”, “doublets” or “compound mutations”, and is a frequent finding (Jacobsen JN et al., Oncotarget. 2018;9:26195–208; Cheng Z et al., Oncogene. 2008;27:4336-43, etc…), not being related with a worse prognosis in patients, treated or not, with tyrosine kinase inhibitors. In our study, three of the patients with compounds mutations in the *EGFR* were within the Non-SM group and had better prognosis, whereas the other patient with “doublets” was in the non-SM group (8 vs. 10%, respectively, without statistically significant differences). However, if considered by the reviewers or editor, a side-by-side table of all the 47 patients with their different mutations can be added to the final version of the manuscript. Moreover, we kindly propose the next graphical representation of table 2, and if considered by the reviewer or editor it can be added to a new version of the manuscript:





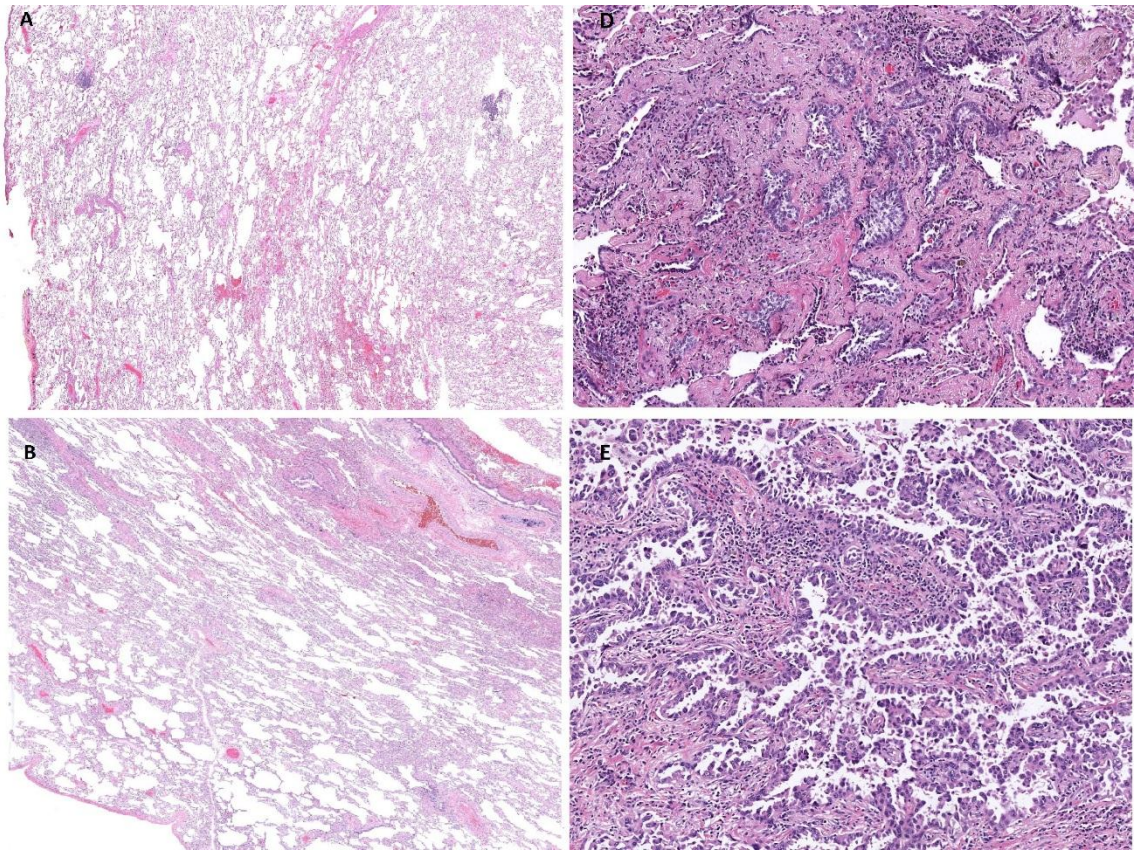
6. *Figure 1 needs a better labeling with clear indication of tumor location. The provided images do not allow a clear appreciation of the staining at the cellular level. This requires more magnification especially for areas with ADCs. Provide semi-quantitative data for mutant KRAS staining. The fact that adjacent cells show positive staining suggests the need for a negative control. This will validate the specificity of the antibody used.*

Figure 1 is an image of the non-tumoral lung tissue where the next-generation PCR and Digital PCR were performed in both a patient with an *EGFR* mutation (A), and a patient with *KRAS* mutation (B). These two patients were in the SM group. Moreover, the 1x magnification has been chosen to show the absolute absence of tumor hallmarks in these samples. We propose, if considered by the reviewers or the editor, to complete the figure 1 with two additional images of the tumoral samples of the same patients with a 10x magnification. You can find below our proposal:

Figure 1. Hematoxylin-eosin staining of two cases with mutations in both non-tumoral lung tissue and lung adenocarcinoma:

(A) non-tumoral lung sample of patient with *EGFR* mutation (1x) and (D) lung adenocarcinoma sample of the same patient (10x)

(B) non-tumoral lung sample of patient with *KRAS* mutation (1x) and (E) lung adenocarcinoma sample of the same patient (10x)



As mentioned in our previous comments, in our study we did not perform immunohistochemistry for the detection of *EGFR* or *KRAS* mutations in the non-tumoral tissue in order to confirm the mutations. We used next-generation real time PCR and Digital PCR. Furthermore, we only used FISH analysis in the initial screening for *KRAS* mutation in the tumor (Sanger sequencing), but for the confirmation analysis we performed CastPCR (TaqMan assay) and Digital PCR (for quantification). However, if considered necessary, a schematic approach of the methodology can be added to the final version of the manuscript to further clarify these points.

7. *Figure 2 has unreadable annotations that need to be fixed*

We thank the reviewer for his/her appreciations. The revised version of the manuscript is an automatically generated version. Below you can see figure 2 with its original quality:

Figure 2. Kaplan Meier curves for (A) time to progression, and (B) disease free survival time. Abbreviations: SM, genetic alterations (*EGFR* and/or *KRAS*) observed both in the

tumor and the non-tumoral lung parenchyma. Non-SM, genetic alterations shown only by the tumor.