Peer Review File

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Reviewer A

Major comments

1. *Given the limited amount of proteins analyzed (92 proteins), authors should comment/discuss that immune-related proteins may not be the only ones of importance in osimertinib-resistance. A more comprehensive protein analysis on solid tissue biopsies could possibly reveal additional or different protein signatures.*

Reply 1: We agree that analysis of more proteins could give additional information and that a comprehensive protein analysis of tissue biopsies would be very beneficial to this study to validate the findings in the blood. However, tissue biopsies are rarely taken at disease progression and we were therefore not able to perform such analysis. Most patients would have a tissue sample from the time of diagnosis however this sample is unlikely to represent the tumor biology at disease progression given the long treatment history of the patients. We still find this point valid and a section has been added to the discussion.

Changes in the text: We added the following sentence to the discussion "Although this study identifies immune response-related proteins to be of importance during osimertinib treatment it is most likely that other protein pathways are also involved in osimertinib resistance which could be identified using a larger protein panel." (Page 10, line 237-239)

2. *The M&M is scarcely described. Authors should at least briefly describe how the plasma samples were processed before PEA analysis and not only reference previous papers. Were plasma simply lysed in a lysis buffer or subjected for extracellular vesicle extraction before lysis? This is important information to understand the sub-biological source of the analyzed proteins.*

Reply 2: We agree that this part of the materials and methods could be described in more detail. We have added some information regarding the sample processing to the revised version of the manuscript. No other processing was performed other than plasma isolation via centrifugation.

Changes in the text: The following sentence "Blood samples were collected in EDTA tubes approximately every 4-6 weeks. Plasma was isolated and stored at -80 °C (29)" was changed to "Peripheral blood was drawn from each patient approximately every 4-6 weeks in 10 mL EDTA tubes and centrifuged within 6 h at 1400 *g* for 15 min at room temperature. Plasma was aliquoted and stored at -80 °C (29)" (Page 5, line 108-110)

3. *According to Figure 2, only one protein (NECTIN4), was upregulated in PD samples. However, in Figure S2 and S3, all seven proteins are listed with a negative median value. Shouldn't NECTIN4 have a positive median value?*

Reply 3: This is a valid point and the reviewer is correct. We made a typo in the NECTIN4 plot. The median difference in NECTIN4 between response and progression is 0.28822 and not -0.28822. This has been changed in revised version of the plots. This also means that a reduction or a low increase in NECTIN4 between response and PD gives a better outcome than NECTIN4 increase following progression. In the original manuscript this conclusion was depicted the other way around where an increase in NECTIN4 was associated with a good outcome. Thank you for pointing this out for us. Furthermore, figure S3 has been incorporated in figure 2 due to the comment below.

Changes in the text: Figure S2 and S3 has changed -0.28822 to is 0.28822 for the NECTIN4 plots.

Minor comments

1. *Authors may want to consider moving some of the supplemental figures to main figures. Currently the main figures contain a total of five panels (separated in four figures). Especially, Figure S3 may be worth more attention.*

Reply 1: In the revised version of the manuscript, we have incorporated figure S3 as part of figure 2. In this process we discovered an error in the original figure S3. We discovered that the p-value of the log-rank test for CD27 (0.0472) was wrong the actual p-value is 0.13 as reported in the new figure 2B. This is an unfortunate error, and as a result, all statistical analyses have been reevaluated which didn't result in any more corrections. This change does not affect the overall message of the revised manuscript.

Changes in the text: The section regarding figure 2 (headline: Discovering differences in circulating proteins) has been changed to accommodate the implementation of figure S3 as figure 2B (Page 8, line 179-185). The legend of figure 2 has also been modified (Page 18, line 475-481). Moreover, because Figure S3 has been moved the old figure S4 and figure S5 have been renamed to figure S3 and figure S4 which has been changed in the revised version of the manuscript.

2. *Row 259 contains symbols instead of a protein name after LY9.*

Reply 2: This has been changed in the revised version of the manuscript

Changes in the text: "错误!未找到引用源" to "Figure S3" (Page 9, line 217)

Reviewer B

Major comments

1. *Line23: I feel that the Running title is slightly different in meaning from the main title. I feel that there is something wrong with "resistance". Please correct it.*

Reply 1: We agree that resistance might not be the correct word in this context and have therefore updated the running title in the revised version of the manuscript

Changes in the text: "Immune proteins predict outcome of osimertinib resistance" was changed to "Immune proteins predict outcome at osimertinib progression" (Page 1, line 17)

2. *Line106-109: "We find that differences～of osimertinib resistance." is the result of this study and not the content of the introduction. Please correct it.*

Reply 2: We agree that this is a result of the study and not an introduction of the study. This has therefore been removed from the revised manuscript.

Changes in the text: Removed the following sentence "We find that differences in proteins related to the adaptive immune system can predict the overall survival (OS) after PD. This highlights that the immune system can also have a role in the development of osimertinib resistance."

3. *Line259: Please correct the non-English characters*

Reply 3: This has been changed in the revised version of the manuscript

Changes in the text: "错误!未找到引用源" to "Figure S3" (Page 9, line 217)

4. *Line274: The small sample size is also an important limitation and should be clearly stated in the Discussion.*

Reply 4: We agree that this is a limitation to the study and this has been added to the discussion in the revised version of the manuscript

Changes in the text: Added the following sentence to the discussion "One of the limitations of this study is the small number of patients used in the survival analysis, where the group median is necessary to distinguish the immune high and low groups." (Page 11, line 275-276)

5. *Line 274: In the Discussion, please describe the relationship between smoking and "immune response" Proteins. We know from this study that smokers have higher expression of "immune response" Proteins. Please provide additional analysis and mention whether smoking history is a confounding factor for the study's conclusion that higher expression of "immune response" Proteins is associated with worse prognosis after osimertinib. Please mention it.*

Reply 5: This is an important an interesting point raised by the reviewer. It is likely that smoking affects the immune landscape of the patients which could also affect the plasma protein levels at PD. We have added a paragraph about this point to the discussion.

Changes in the text: Added the following section to the discussion "Interestingly this study demonstrates that patients with high amount of immune response-related proteins are more likely to have a smoking history compared to patients with a low amount of immune response-related proteins (Table 1). This supports the idea of smoking affecting the immune landscape of lung cancer patients (37) and future studies is needed to evaluate the involvement of smoking and the immune system at osimertinib progression." (Page 10, line 242-246)

6. *Line 274: At the end of the Discussion, it would be even better if you could address how the results of this study may benefit patients with EGFR mutation-positive lung cancer in real-world clinical practice.*

Reply 6: In the discussion we put this study into the context of similar published studies. A new paragraph has been added to this section which elaborates more in detail how this study can be applied in a clinical setting and how it cooperates with ongoing clinical trials.

Changes in the text: Added the following paragraph to the discussion "Combined, these studies indicate that some patients with tumor progression on osimertinib could benefit from immune checkpoint inhibitor therapy. This is currently being investigated in combination with chemotherapy in the KEYNOTE-789 and CheckMate722 phase III clinical trials. Evaluating the immune-related proteins in plasma at PD on osimertinib, could potentially help to stratify which patients would benefit from immunotherapy. " (Page 11, line 269-274)

7. *As for Figure S4, I think it is an important Figure, so how about it as Figure 5? Please consider.*

Reply 7: Figure 4 is based on the plasma level of proteins related to the immune-response and figure S4 is based on the plasma level of proteins related to the adaptive immune response. In the PEA panel we have used, only one gene is different between these two gene groups (FASLG). We therefore believe the two figures are somewhat redundant and the small differences seen in the analyses could be by chance. In order to simplify the message of the study we only display the figure with the immune-related proteins in the main text, whereas the figure with the proteins related to the adaptive immune system is kept in the supplementary files

Changes in the text: None.

Reviewer C

Major comments

1. *In figure 2 and table 2, the log2FCs of the seven proteins were very small and there were no remarkable differences between median at response and at PD. The reviewer concerned that the differences might be by chance. The authors should discuss this point.*

Reply 1: We agree that the log2FC are small, however we believe the differences could still be of clinical value. Because the changes of the individual proteins are small, we use the combined sNPX values to get a bigger picture of the overall level of immune related proteins. In order to avoid false positive results in the statical analysis we use multiple-testing (FDR) adjusted q-values which reduces the likelihood that the differences are detected by chance. This has been further clarified in the statistical analysis section.

Changes in the text: The following sentence "Following false discovery rate (FDR) adjustment (35) two-tailed *q-value* < 0.05 were considered significant" has been changed to "To correct for multiple testing, false discovery rate (FDR) adjustment (35) was performed where a two-tailed *q-value* < 0.05 were considered significant." (Page 6, line 148-149)

2. *The authors should describe the way how they calculate the summarized NPX values for proteins associated with the GO terms and explain the validity of the method.*

Reply 2: We believe that proteins can have overlapping functions and therefore adding the proteins together gives a better picture of the immune status in the patient. We did this to get a combined estimate of the level of immune or adaptive-immune related proteins. We have added a sentence in the revised version of the manuscript which more clearly describes how the sNPX values were calculated.

Changes in the text: Added the following sentence "sNPX values are calculated by adding the NPX values for individual proteins related to a specific GO term together for each patient." (Page 6, line 142-143)

3. *Authors have indicated that a lower amount of circulating immune response proteins at PD is associated with OS after PD. Considering prognosis after osimertinib treatment, patients characteristics at PD of osimertinib is important. Although authors have shown the demographics of the patients at osimertinib start, it seems insufficient. At osimertinib achieving PD, were there any differences in patient characteristics between low and high group? If authors collected, please describe these informative data.*

Reply 3: We agree that the data at PD would be the most informative give the primary outcome is to investigate the OS following PD. Unfortunately, these data are not available at PD but only at the start of osimertinib treatment and are therefore not reported.

Changes in the text: None.

4. *In this study, almost osimertinib has been utilized as second-line. The authors should clear the type of first line therapy because the type of first line therapy may influence on this result*

Reply 4: We agree that this should be clearer. All patients treated with osimertinib in second-line treatment received erlotinib as first line of treatment. This note has been added to the revised manuscript.

Changes in the text: Added the following sentence to methods "All patients receiving osimertinib as second-line of treatment received erlotinib as first-line of treatment." (Page 4, line 95+96)

5. *The authors should add the time period of patient enrollment (e.g; from when to when) in methods*

Reply 5: This has been added in the revised version of the manuscript

Changes in the text: The following sentence "The study was conducted at The Department of Oncology, Aarhus University hospital, and included patients from four oncology departments in the western part of Denmark" has been changed to "The study was conducted at The Department of Oncology, Aarhus University hospital, and included patients from four oncology departments in the western part of Denmark between August 2014 and December 2018" (Page 4, line 89+90).

6. *In methods, the authors should clearly state what the primary outcome in this study is.*

Reply 6: We agree that this was not clear in the original manuscript and has been updated in the revised version

Changes in the text: Added the following sentence to methods "The primary outcome of this study was to evaluate the OS following PD on osimertinib" (Page 5, line 99+100)

7. *The authors mentioned adaptive immune response proteins at PD is predictor for OS after PD with osimertinib. The reviewer concerned this is just malignant related factors when tumor re-growth, because adaptive immune response is supposed to associate with the inflammation of whole body. The authors should discuss this point.*

Reply 7: We agree that this present study does not provide any evidence in regard to the origin and function of the adaptive immune response proteins detected at PD. These proteins could be secondary and simply caused by tumor regrowth. We wanted to identify a prognostic factor at PD which could explain the outcome of patients following PD on osimertinib. Future studies will need to clarify the origin of the proteins as well as the temporal relationship between immune activation and tumor progression in order to identify a causal link between the two. A comment about this has been added to the discussion in the manuscript

Changes in the text: Added to the discussion: "Furthermore, future studies could address the causal link and temporal relationship between immune-related proteins in plasma and tumor progression on osimertinib." (Page 10, line 239-241)

8. *The authors should mention the efficacy of immunotherapy after osimertinib resistance, related to activation of adaptive immune response proteins.*

Reply 8: We agree that some patients treated with osimertinib could benefit from immunotherapy following disease progression. Potentially monitoring the levels immune related proteins during treatment of osimertinib could help identify which patients that would benefit from immunotherapy. A section has been added to the discussion regarding this topic in the revised version.

Changes in the text: Added the following section to the discussion "Combined, these studies indicate that some patients with tumor progression on osimertinib could benefit from immune checkpoint inhibitor therapy. This is currently being investigated in combination with chemotherapy in the KEYNOTE-789 and CheckMate722 phase III clinical trials. Evaluating the immune-related proteins in plasma at PD on osimertinib, could potentially help to stratify which patients would benefit from immunotherapy." (Page 11, line 269-274)

Minor comments

1. *Please define the abbreviations of "FC" at the first use in the manuscript.*

Reply 1: This has been addressed both in the main text and the legend of figure 2 and table 2

Changes in the text: "Although not statistically significant, CEACAM5 had the highest Log2FC (1.55, equivalent to 2.93 increase in linear values)" Changed to **"**Although not statistically significant, CEACAM5 had the highest Log2 fold change (Log2FC) (Log2FC = 1.55, equivalent to 2.93 increase in linear values)" (Page 8, line 186). "Log2FC: Log2 fold change" has been added to legend of figure 2 (Page 18, line 480) and table 2.

2. *The definition of PFS, OS, and OS after PD is unclear. Authors should show how they define the period of these treatment outcomes clearly.*

Reply 2: We have revised the methods section and added clearer definitions of OS, PFS and OS after PD to this section.

Changes in the text: We have added the following sentence **"**PFS was defined as the time from osimertinib start until PD. OS was defined as the time from osimertinib start until death or censoring of data, whereas OS after PD was defined as the time from PD until death or censoring of data.**"** (Page 5, line 100-102)

3. *In table 2, "-019" is a typo*

Reply 3: This has been changed in the revised version of table 2.

Changes in the text: Table 2 -019 to -0.19

4. *In Figure 1, 24 patients with lack of information. Response: BS max 2 months "bbefore"… is a typo.*

Reply 4: You are right. This has been changed in the revised version of figure 1.

Changes in the text: Figure 1 "bbefore" to "before"

5. *In line 258-259, "Adaptive immune response" (CD27, CD70, CXLC13, ICOSLG,LY9, 错误!未找到引用源。), .. Is "错误!未找到引用源" is a typo.*

Reply 5: This has been changed in the revised version of the manuscript

Changes in the text: "错误!未找到引用源" to "Figure S3" (Page 9, line 217)

6. *Page2, Line63-66: The authors mentioned the frequency of EGFR mutatnt NSCLC patients, but this information is only Caucasian, but not east Asian. They should add a postscript.*

Reply 6: Given this study takes place in Denmark we have focused on the Caucasian frequency of EGFR mutations. This has been specified in the revised version of the manuscript

Changes in the text: "Ten to 15 percent of non-small cell lung cancer (NSCLC) adenocarcinomas are caused by activating mutations" Changed to "Ten to 15 percent of non-small cell lung cancer (NSCLC) adenocarcinomas in Caucasians are caused by activating mutations" (Page 3, line 46)

Re-review comments

Reviewer A:

The authors have addressed my comments. I recommend the manuscript to be accepted for publication. The NECTIN4 plot in Figure S2 is still listed with a negative median value and should be changed to a positive median value.

Reply: This has been done in the revised version of the supplementary figures

Reviewer B:

I read this paper with great interest.
We also appreciate the appropriate responses to the points raised by the reviewers.
Please confirm one additional point.
Regarding Reviewer A's comment 3
Figure S2. shouldn't the median value of NECTIN4 also be changed from -0.28822 to 0.28822?

Reply: This has been done in the revised version of the supplementary figures