TRANSLATIONAL LUNG CANCER RESEARCH

Peer Review File

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Reviewer A

In this study Christensen Wen and colleagues tested the prognostic value of methylated HOXA9 in plasma samples of late stage (III and IV) NSCLC patients on overall survival (OS) and progression free survival (PFS). The authors analyzed cfDNA from plasma samples from a reasonably large retrospective cohort of 228 NSCLC patients and found significant negative association between the measurable methylated HOXA9 DNA in plasma and OS or PFS. This indicates that the detection of methylated HOXA9 in plasma could be potentially used as a prognostic factor in late stages of NSCLC. Although multiple studies have been published about HOXA9 methylation in cancers, this study appears to be the first one about the potential use of HOXA9 methylation in cfDNA as a prognostic factor for late NSCLC stages. Overall, the study is well written, and the authors discuss most of the study limitations. There are several points including the methods and figures that should be amended, see below:

Specific points:
To guarantee reproducibility, the Methods should contain more details, specifically:
Comment 1: How the blood was processed to obtain plasma? (how many spins, times, speeds (gs))
Reply 1: We have now specified how the blood was processed to obtain plasma.
Changes in the text: Page 9, line 1: Two 9 ml peripheral blood samples were collected into EDTA-containing tubes and centrifuged at 2,000 g for 10 minutes.

Comment 2: How much converted DNA was analyzed by ddPCR?
Reply 2: We use all the DNA from the patient's sample for the analysis. We use 4 ml plasma obtained as described in reply 1, and the purified DNA is eluted in 60 ul and then concentrated to a final volume of 20 ul. We do not measure the concentration of DNA in the sample and then use a specific amount of DNA as some laboratories do, e.g. for next generation sequencing.
Changes in the text: Page 9, line 13-15: The purified DNA was concentrated to a final volume of 20 µl. All of the purified DNA was bisulfite converted using the EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, California, USA) as recommended by the manufacturer.

Comment 3: How much DNA from healthy donors was used as non-cancer control?
Reply 3: We use 20 ul of purified DNA from healthy donors, which corresponds to around 20 ng of DNA. The DNA is bisulfite converted along with the patient sample. We have added a detailed description of the controls to the supplementary appendix.
Changes in the text: Page 9 lines 18-19: a pool of lymphocyte DNA from healthy donors (20 µl

corresponding to approximately 20 ng) as non-cancer control,

Comment 4: How much universal methylated DNA was used as a positive control?
Reply 4: We use the Universal Methylated Human DNA Standard from Zymo Research as described. We mix it with donor DNA and water. The content is now described in the supplementary appendix.
Changes in the text: Page 23, lines 13-19: Positive control:
Universal Methylated Human DNA Standard, 2 µl (DNA concentration 250 ng/µl, Zymo Research, Irvine, California, USA), healthy donor lymphocyte DNA, 199 µl (DNA concentration approximately 20 ng/µl) and water 800 µl.
For each reaction, 20 µl of this mixture was added and bisulfite converted in parallel with the patient samples.

Comment 5: The ddPCR itself should also be described in more detail, when one follows the references 24 and 25 sufficient amount of details to replicate the assay could not be found there.
Reply 5: We have added more details including the PCR conditions to the supplementary appendix in order to make the droplet digital PCR assay replicable.
Changes in the text: Page 23, lines 21-23 (including Table S1). Table S1: PCR conditions for the methylation specific droplet digital PCR assay

Steps	Temperature	Time
Step 1	95ºC	10 minutes
Step 2: 44 cycles	95ºC 56ºC	15 seconds 1 minute
Step 3	98ºC	10 minutes

Machine: Veriti Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Foster City, California, USA).
Ramp rate: 1.5ºC/second.

Comment 6: The Figure 3 (KM plots) does not show 95% CIs, although the figure legend refers to them. The figure should be updated to show 95% CIs.
Reply 6: The reviewer is absolutely correct. We have now updated the figures to show the 95% confidence intervals.
Changes in the text: Figure 3 A-D has been updated to show the 95% confidence intervals, and the graphs are now in color.

Comment 7: The supplemental figures S1-S6 are also missing to show 95% CIs.
Reply 7: This is also correct, and the figures have been updated.

Changes in the text: Figures S1-S6 have been updated to show the 95% confidence intervals.

Comment 8: Table 1 – it would be more appropriate to show in the second column data for total of 228 patients that provided samples, excluding three subjects that did not provide samples and therefore could not be analyzed, rather than showing data for all 231 "baseline" subjects.
Reply 8: Yes, we discussed this issue in the author group before submitting the manuscript. In principle, it would be more correct to leave them out. However, the three patients who did not provide samples at baseline (or in which the baseline analysis failed) were included in Table 1 (in the column 'Total') because they all provided samples after the first cycle of treatment. We found it important to keep the characteristics of these three patients in Table 1, since otherwise they would not be reported at all.
Changes in the text: No changes.

Comment 9: The suggestion to use a group of untreated patients in a prospective study as a control group (discussion page 11 line 12) is scientifically sound, however, questionable from an ethical point of view.
Reply 9: Yes, that would be the scientifically ideal scenario, but the reviewer is correct in observing that it would be ethically questionable. We suggest instead a randomized trial to evaluate the use of methylated HOXA9 for evaluation of treatment reconsideration. This would allow us to investigate whether methylated HOXA9 is clinically useful for treatment reconsideration.
Changes in the text: Page 15, lines 5-6: This remains to be elucidated in a prospective trial ideally randomizing patients to either standardized follow-up with CT evaluation or follow-up including metHOXA9 status for treatment reconsideration.

Comment 10: Although the quite long (up to 8 years) plasma storage is discussed, it was published previously (PMID: 16368947, DOI: 10.1093/jnci/dji432) that the DNA is lost/degraded during plasma storage, and the average decrease in cfDNA amount is about 30% per year of storage. The reviewer has similar experience, virtually no cfDNA yield after 10 years of plasma storage. Have the authors found any correlation between their DNA yields and plasma storage times?
Reply 10: Yes, you are correct, and we are aware of this issue. In our experience, however, the level of cell free DNA does not decrease as steeply as reported above. We have measured total cell-free DNA in the form of the β2 microglobulin gene in the present patient cohort. We find a median 2825 copies/ml (mean 4920 copies/ml, range 279-106656 copies/ml) across the 228 baseline samples. We have measured the level of the β2 microglobulin gene in another cohort of patients (advanced or recurrent lung cancer eligible to receive check-point inhibitor immunotherapy at any treatment line) with samples collected from September 2017 to November 2019 and analyzed in March 2020 (storage time up to 2.5 years). In the baseline samples from these 80 patients we found a median 2664 copies/ml β2 microglobulin (mean 3674 copies/ml, range 572-17988 copies/ml). These results

are similar to the findings in the present study. We believe that the long storage time did not affect the samples to a degree that would significantly affect the results, although lower storage time would always be preferable. We have added a paragraph about cell free DNA yield to the supplementary appendix.

Changes in the text: Page 15, lines 14-16. However, it was previously reported that the loss of cell free DNA in frozen plasma is about 30% per year of storage (REF to the study mentioned by the reviewer). We believe that the storage time did not affect our samples to a degree that would significantly affect our results, although shorter storage time would have been preferable.

Page 24, lines 2-5: Cell free DNA yield:

We have measured total cell-free DNA in the form of the β2 microglobulin gene in the present patient cohort. We found a median 2825 copies/ml (mean 4920 copies/ml, range 279-106656 copies/ml) across the 228 baseline samples.

Reviewer B

The authors explore the prognostic impact of circulating metHOXA9 in patients with advanced NSCLC. They show that detection of metHOXA9 at baseline is a negative prognostic factor in patients with NSCLC stage III and IV, and that the effect is enhanced after the first treatment cycle. I found this article very interesting and useful, with great translational potential. The only thing that I found as a problematic was language, it was hard for me to follow the text! I would recommend to read the manuscript again (native speaker) and correct the "clumsy sentences" (the longer you are alive despite your biomarker status, the longer you will likely keep on living, etc.)

Comment 1: (Above)

Reply 1: Thank you for your time and effort reviewing the manuscript. We have carefully re-read the manuscript and asked for professional advice on the language issues. Please refer to the revised manuscript for all corrections. The specific sentence used as an example above has been rephrased.

Changes in the text: Page 14, lines 7-8. Detectable metHOXA9 has a certain impact on the prognosis after the first treatment cycle, and then it gradually decreases.