Peer Review File

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Reviewer A

Authors present a manuscript investigating the role of methylation profiling in the diagnosis of lung squamous cell carcinoma. They demonstrate differential methylation profile for tumour, peritumoural and normal tissue and a diagnostic profile using cell free DNA for stages I-III. Both show good performance in differentiating tumour from peritumoural and/normal tissue.

Comments:

Overall authors present a well designed and clearly written study.

Comments 1.The limitations of the study include its small sample size and a single centre study. This is stated in the manuscript and they acknowledge that a large multi-center study is required to fully validate their findings.
Reply 1: Thanks for your opinion. The sample size is indeed a limitation of our study and we have listed in the manuscript. We plan to reanalyze our data in the further when a large-scale number of sample have been recruited.
Changes in the text:-

Comments 2. There has been significant work on methylation biomarkers in LUSC e.g. PMID 31546933,PMID: 35356464. The introduction and discussion of the manuscript do not address this. Authors also do not adequately discuss how their findings fit in the overall body of the literature and do not explicitly state how their contributions are distinct from what is already in the literature.
Reply 2: Thanks for your kind suggestion. Here we have updated our discussion in the revised manuscript referred to line 326-330 and line 333-335
Changes in the text：page10-11；line 326-330 and line 333-335

Comments 3. An additional limitation is that only a training cohort was used to develop the diagnostic bioinformatic tools. A well grounded study requires an independent validation cohort or at least cross-validation study needs to be done.
Reply 3: The validation cohort is indeed a limitation of our study. We plan to reanalyze our data in the further when validation cohort has been recruited to verify our findings.
Changes in the text：-

Comments 4. The assay is most important clinical use would be for the detection of early stage LUSC. The ROC curve was validated for stages including stages I-III. It would be interesting to know the performance in early stage LUSC.
Reply 4: We thank the reviewer for the kind suggestion. It's really important to diagnose early stage in clinical practice. Here we added a subgroup analysis for selecting these stage I LUSC patients and 46.2%(6/13) have been tested as patients with the CD-score model, which means

a sensitivity of 46.2% in stage I LUSC. Here we know the limitation of this result as its small size, and for the future we may recruit more participants to update its performance in prospective cohorts.
Changes in the text: -

Reviewer B

General remarks
The authors examined the tumor tissue, paired peritumoral tissue, distant normal tissue and blood samples from 49 squamous cell lung cancer patients and compared the methylation profiles with plasma samples from healthy donors. they find differentially methylated blocks in tissue and blood samples and conclude from their studies that it is possible to differentiate the analyzed tissues from each other.

Specific comments

Comments 5:line 36"...serum samples...."the application of Streck tubes for blood drawing results in plasma but not serum! (see line 40ff),
Reply 5: Thank you for the correction. We have corrected it in the revised manuscript.
Changes in the text:see page 2,line 36 .

Comments 6: lines 113/114"and the ability to overcome various limitations of clinical practice due to tumor heterogeneity", the use of cell-free DNA does not solve this problem, i.e. tumor heterogeneity, in contrast it might be even more difficult to detect/quantifiy tumor associated alterations due to genetic material not only released from the tumor but from metastastatic lesions as well, also, in most patients the amount of cell-free DNA originates in normal, i.e. healthy cells,
reply 6：Thanks for your suggestions. Yes, we admit that it is difficult to detect or quantify tumor associated alterations in liquid biopsy as you have pointed out the reasons. So this study just focus on DNA methylation and we also sequenced the tumor samples, which helps us to confirm what we have found in liquid biopsy was from tumor cells, this is recently called tumor informed method. However, the limitations in line 113/114 related to tumor heterogeneity, we meant that in tissue level we will need samples from each lesions to quantify its heterogeneity. But in blood there will be tumor molecular from all the lesions. Also, we admit its challenge in liquid biopsy to detect tumor signals.
Changes in the text：-

Comments 7: lines 116-9"A previous study has demonstrated the potential clinical utility of cfDNA methylation profiling for the sensitive detection, monitoring and molecular subtyping of patients with small-cell lung cancer [16].LUSC belongs to the group of NSCLC, there are papers published in which methylome profiling for this group is shown and these paper should be referenced instead of Chemi et al (dealing with SCLC tumors) or in addition to Chemi et al,
Reply 7: Thank you for the correction.
Changes in the text: see the page 4 , line118-119.

Comments 8: lines 135-7 healthy volunteers are not an adequate control group, it would have been better to include patients with benign lung diseases instead, additionally the mean (!) age of the patients was 63 years vs median (!) age of 44 years for the control group, is there a specific reason for using mean vs median age?
Reply8: We thank the reviewer for the kind suggestion. We will consider adding patients with benign LUSC data when we preparing more mature data. And the patients age information using “mean age” is a clerical error， it should be “median age”.
Changes in the text: see page 18, line 478 -table1, line “Age”.

Comments 9: line 130 and 139
the study was performed from June 2016 to August 2020 and approved by the Clinical Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University in 2023 {??}, does that mean that the patients gave their informed consent 3 to 7 years after treatment?
Reply9: Thanks for your opinion. In our hospital there is a research sample bank. These samples have signed informed consent forms for research in advance. And all the doctors in our hospital can design a new research project and submit it to the sample bank for approval. And this sample bank did speed up most of our translational studies a lot. That’s why the study approval comes after sampling.
Changes in the text：-

Comments 10: line 253-8 was there any correlation between the quantity of tumor cells vs normal/healthy cells in tumor tissue samples and the intensity of methylated blocks? in addition, was there any correlation between the quantity of tumor cells vs normal/healthy cells in the peri-tumor samples and the intensity of methylated blocks? in general: what was the percentage of tumor cells in tumor tissue and in peri-tumoral tissue samples?
Reply10: Thank you very much for sharing your opinion. Yes， there is some correlation between the quantity of tumor cells and normal cells in tumor tissue samples and the intensity of methylated blocks, but this correlation may be influenced by factors such as the type, stage, location, etc. of the tumor. In general, the percentage of tumor cells in tumor tissue and in peri-tumoral tissue samples may vary depending on the type and stage of the cancer, the presence of immune cells and stromal components, and the method of tissue preparation and analysis. Some studies have reported that the percentage of tumor cells in tumor tissue can range from 10% to 90%, and that the percentage of tumor cells in peri-tumoral tissue can range from 0% to 50%. The peri-tumoral tissue in this study all have been confirmed with 0% tumor cells.
Changes in the text：-

Comments 11: line 259-67
how good is your approach to differentiate between tumor patients and patients with a benign disease (which would be closer to the clinical reality)?
Reply 11: Thank you very much for sharing your interesting opinion. The design and purpose of our study was referred to benign lesions. We mostly wanted to detect tumor signals from para-tumor, which have been confirmed with no tumor cells in pathological imaging. Perhaps

what we have found may draw a clue for benign disease differentiation and propose a new study if there are applicable benign samples.
Changes in the text：-

Comments 12: line 281-92, 348
discriminating tumor patients from healthy peope does not reflect the clinial reality (see above), much more important is the discrimination of tumor patients from people with a benign disease, what is the congruence of the methylation profiles between tumor tissue and blood samples from LUSC patients?
Reply 12: We thank the reviewer for the kind suggestion. The design and purpose of our study was not and does not require comparison with benign lesions. And because of sample limitation we did not have the capacity for this kind of study. Perhaps future studies can explore the differences between tumors and benign lesions. For the blood samples and LUSC tissues, they expressed different methylation profiles as we can see in Figure1B. And the majority of differentially methylated regions in tumor tissues are different from signals from blood sample. In the forth sections of our results(line 277), we compared there intersection and only found 3 methylated blocks shared.
Changes in the text:-

Comments13: line 303-4
are the two subgroups clinically different? if yes, pls specify,
Reply 13: Thank you for your suggestion. We have compared their clinical characteristic including age, gender, smoking history, stage, differentiation level, tumor size and found no significant difference.
Changes in the text:-

Comments 14: line 325-7
"In addition, we found that the field cancerization blocks were independent of the clinical factors, supporting potential role of methylation markers in the LUSC diagnosis"
as long as this statement is correct, the described method should be able to differentiate between sick {benign disease) and tumor patients
Reply 14：Thanks for your kind opinion and we admitted this suggestion. However, there are some limitations for benign samples in the sample bank. So we just started this project focusing on patients. Certainly, it is much more important for benign disease separation in clinical practice. Hoping this research can help to find some clue for LUSC diagnosis in the future.
Change in the text:-