

Peer Review File

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Reviewer Comments

This is an interesting and timely paper with the potential to expand our knowledge of potential prognostic biomarkers for HCC. However, there are a number of major issues that need to be addressed before publication.

Major points:

Comment 1:

Mediation analysis: How can “gene expression” be considered an independent mediator in this scenario? Surely HCC is directly dependent on “gene expression”? Also the 24.8% prognostic effect of DNA methylation (line 229) implies that in 75% of cases the effect of methylation on HCC prognosis is not mediated by gene transcription. How do the authors rationalise these results?

Reply1:

We thank the reviewer for the precious comment. We agree with the reviewer that gene expression is an independent factor affecting HCC outcomes. However, in this study, we consider “gene expression” as an indirect mediator of DNA methylation affecting overall survival of HCC. DNA methylation demonstrated significant impact on overall survival (HR=2.54, $P=8.65E-07$). The previous literature indicated that DNA methylation may impact the cancer outcomes via mediating their corresponding gene expression [1]. Therefore, we proposed that ferroptosis related gene DNA methylation and corresponding gene expression may follow the similar model in HCC. Regarding the issue of “24.8% prognostic effect of DNA methylation was mediated by gene expression”, most of methylation’s effect may act beyond gene expression, but gene function [2]. Although CGI (CpG Island) methylation can result in stable repression of the linked gene, few CGIs change DNA-methylation state during normal development, like promoter methylation is required for regression of a set of germline-specific genes [3]. Therefore, we proposed that the 75% of cases the effect of methylation on HCC prognosis is mediated by other factors, like promoter methylation induced gene function alterations. We justified this part in the discussion. This part has been revised and highlighted in the manuscript.

Changes in the text:

We have modified our text as advised at Page 10, line 236-239.

Comment 2:

ferroptosis-related epigenetic score – can this be explained better? Where does this originate from? Can the authors explain why known ferroptosis relevant genes such as ACSL4 are not included in this score?

Reply2: We are sorry for the confusion. To make it clear, we modified ferroptosis-related epigenetic score as ferroptosis-related methylation score, which originated from validated significant CpG sites associated with overall survival of HCC. The 5 CpG sites were further identified using SIS method and stepwise regression (cg02916418, cg05373863, cg13028471, cg07137701, cg15044146, details in **Table S2**). We then constructed the ferroptosis-related epigenetic methylation score using 5 CpGs and their regression coefficients: $\text{Score}_{\text{methylation}} = -2.69 \times \text{cg02916418} - 6.69 \times \text{cg05373863} - 12.15 \times \text{cg13028471} - 29.88 \times \text{cg07137701} + 5.97 \times \text{cg15044146}$. We selected gene list based on published literature [4] (**Table S1**). ACSL4 was in our gene list, but unfortunately, it was not significant in predicting overall survival of HCC, thus it was not appeared in the score.

Changes in the text:

We have modified our text as advised at Page 6-7, line 151-156.

Comment 3:

Scoregene and scoremethylation on page 4. The information is presented in a difficult to read, condensed, equation format. Could the readability of this section be improved and more context provided to explain these equations and choice of genes?

Reply3: We thank the reviewer for the suggestion. The part has been throughoutly revised, all the changes have been highlighted in the manuscript. We hope the revised part has been significantly improved according to the reviewer.

Changes in the text:

We have modified our text as advised at Page 6, line 136-148.

Comment 4:

Line 240. “Remarkable performance”. This is not correct. AUC values of 0.6 – 0.75 are normally only considered to demonstrate poor to moderate performance.

Reply 4: We thank the reviewer for the comment. We agree with the reviewer for this incorrect expression. We revised the “Remarkable performance” to “moderate performance” in the manuscript.

Changes in the text:

We have modified our text as advised at Page 11, line 248.

Minor points.**Comment 5:**

“sure independence screening” is repeated in the abstract. Also I am not sure what the authors mean by this phrase?

Reply 5: We are sorry for the typo. Sure independence screening is a method applied in the establishment of multi-CpG-based classifier predicting overall survival based on LASSO Cox penalized regression, using the R package “SIS”.

Changes in the text:

We have modified our text as advised at Page 1, line 26.

Comment 6:

There are many grammatical errors which should be addressed in order to improve the readability of the manuscript.

Reply 6: We are sorry for the grammatical errors in the manuscript. We have throughoutly revised the manuscript, and we hope the readability has been significantly improved.

Changes in the text:

We have modified our text as advised and highlighted the changes in the text.

Comment 7:

c. “inconsistence” – I don’t understand the use of this word here. Perhaps it is an error?

Reply 7: We are sorry for the mistake here. It shall be “inconsistent” instead of “inconsistence”.

Changes in the text:

We have modified our text as advised at Page 10, line 222.