Peer Review File

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Reviewer A

In this manuscript, the author aims to create a novel signature based on fatty acids metabolism-related genes (FAMRGs) to predict prognosis in patients with early-stage lung squamous cell carcinoma (LUSC).

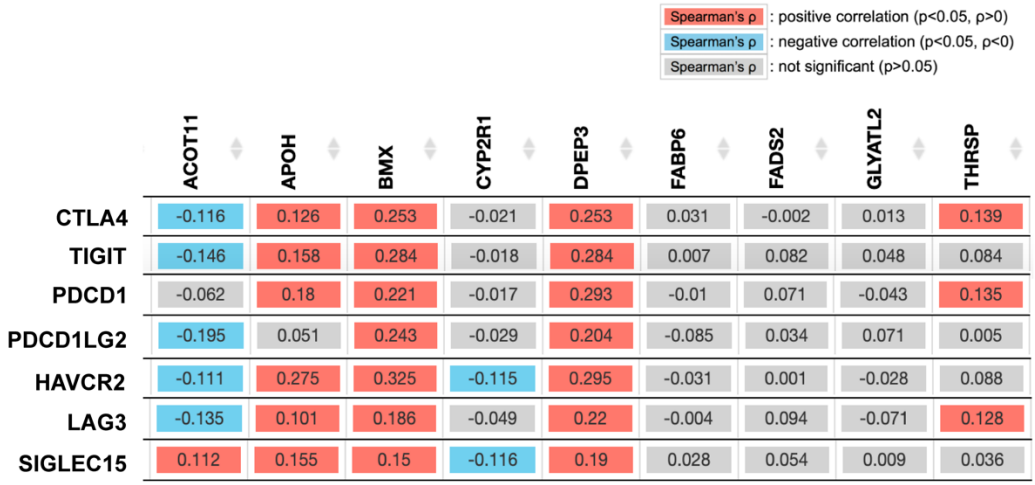
The study's methodology is clearly outlined by presenting a flowchart that guides the research process. The study employed a comprehensive approach, utilizing multiple datasets (TCGA and six independent GEO datasets) for model establishment and validation. The validation of the prognostic model in six independent GEO datasets adds credibility to the generalizability of the findings. The use of risk scores, AUC curves, and Cox regression analyses strengthens the statistical rigor of the study.

The statements are generally well-presented, with a logical flow of information. However, the correlation between high-risk scores and upregulated immune checkpoint genes could benefit from more specific details on the genes involved.

Reply: We sincerely appreciate the valuable advice from the reviewer, and we have incorporated the suggested correlation analysis between gene expression and immune checkpoint genes in our study. Our results consistently revealed that the high-risk group, as defined by fatty acids metabolism-related genes (FAMRGs), exhibited elevated expression several immune checkpoint genes such as CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2, SIGLEC15, and TIGIT. These results illuminate the possibility that early-stage LUSC patients with high-risk scores may manifest a more robust response to therapies targeting the mentioned immune checkpoints. Moreover, our investigation unveiled that patients with high-risk scores also displayed increased expression of the human leukocyte antigen (HLA) genes. Considering the central role of HLA molecules in immunity, variation at the HLA loci could differentially affect the response to immune checkpoint inhibitors (ICIs). Notably, previous studies have associated the loss of heterozygosity at the HLA-I locus (HLA-LOH) with immune resistance in non-small cell lung cancer (NSCLC). Consequently, enhancing the expression of HLA may hold the potential to augment T immune cell activities and improve the response to immunotherapy in NSCLC. In addition, we have provided associations between the expression of the nine FAMRGs-based signature genes and immune checkpoint genes in TCGA-LUSC dataset. As shown in revised Figure 1, the expression of BMX, DPEP3, and APOH exhibited positive correlations with various immune checkpoint genes expression. Conversely, ACOT11 expression was negatively correlated with CTLA4, TIGIT, PDCD1LG2, HAVCR2, and LAG3 expression, while CYP2R1 showed negative correlations with HAVCR2 and SIGLEC15. These nuanced correlations suggest that the FAMRGs-based signature may not influence immune response through a single gene, further highlighting the complexity of the regulatory

network. These additional findings significantly contribute to our understanding of the immune landscape shaped by FAMRGs in LUSC, offering potential avenues for more targeted and effective therapeutic interventions.

Changes in the text: see Page 7, lines 265-281



	ACOT11	APOH	BMX	CYP2R1	DPEP3	FABP6	FADS2	GLYATL2	THRSP
CTLA4	-0.116	0.126	0.253	-0.021	0.253	0.031	-0.002	0.013	0.139
TIGIT	-0.146	0.158	0.284	-0.018	0.284	0.007	0.082	0.048	0.084
PDCD1	-0.062	0.18	0.221	-0.017	0.293	-0.01	0.071	-0.043	0.135
PDCD1LG2	-0.195	0.051	0.243	-0.029	0.204	-0.085	0.034	0.071	0.005
HAVCR2	-0.111	0.275	0.325	-0.115	0.295	-0.031	0.001	-0.028	0.088
LAG3	-0.135	0.101	0.186	-0.049	0.22	-0.004	0.094	-0.071	0.128
SIGLEC15	0.112	0.155	0.15	-0.116	0.19	0.028	0.054	0.009	0.036

Revised Figure 1. Exploring associations between FAMRGs expression and immune checkpoint genes in TCGA-LUSC dataset using TIMER 2.0 (http://timer.cistrome.org/). Red frames represent positive correlation ($p < 0.05$), blue frames represent negative correlation ($p < 0.05$), and gray frames represent not significant ($p > 0.05$).

The correlation between high-risk scores and increased immune cell infiltration and inflammatory activity is mentioned, but the mechanisms underlying this correlation are not explored or discussed.

Reply: We appreciate the suggestion from the reviewer. Our study indeed revealed a noteworthy correlation between high-risk scores and an augmentation in immune cell infiltration, accompanied by heightened inflammatory activity. To elucidate the underlying mechanisms, we delved further into our findings. Our investigation demonstrated an upregulation of immune-related genes, particularly those associated with human leukocyte antigen (HLA) and immune checkpoint genes, in the high-risk group. Notably, immune functions such as CCR, HLA, parainflammation, Type-II-INF-Response, MHC-class-I, and Type-I-INF-Response were markedly elevated in this cohort. These results strongly imply intrinsic differences in tumor immunogenicity among risk groups defined by fatty acids metabolism-related genes (FAMRGs). Furthermore, emerging evidence suggested a potential link between FAMRGs and immune escape, immune tolerance, and antigen presentation loss. Specifically, the dysregulation of immune checkpoint molecules and the modulation of antigen presentation pathways were identified, supporting the notion that FAMRGs play a pivotal role in shaping the tumor immune microenvironment.

Moreover, through Gene Set Variation Analysis (GSVA), we identified the NOD-like receptor signaling pathway as a major player in high-risk group. Previous research has highlighted the involvement of this pathway in the inflammation-associated tumorigenesis, angiogenesis,

cancer cell stemness and chemoresistance. Based on these findings, we propose a hypothesis that FAMRGs may contribute to early-stage LUSC tumorigenesis by regulating the tumor immune microenvironment particularly through the modulation of NOD-like receptor signaling pathway.

Changes in the text: see Page 9, lines 339-356

Addressing the study's limitations and suggesting potential avenues for future research would provide a more comprehensive view of the findings.

Reply: We thank for your valuable comments. We add the limitations of this study and potential avenues for future research. Although the FAMRGs-based signature proves effective as a prognostic marker and emerges as a potential therapeutic target in early-stage LUSC, this study still had some limitations. Firstly, our study was based on some retrospective datasets, and a prospective study to validate the utility of this FAMRGs-based signature will be necessary. Secondly, the conclusion drawn from our integrated analysis of clinical samples obtained from public databases, and the findings need validated through new methodologies and the inclusion of fresh specimens. Thirdly, while we indirectly assessed the ability to predict responses to immune, chemo, and targeted therapies, a more in-depth exploration of the underlying mechanisms of the signature in predicting treatment responses is warranted.

Changes in the text: see Page 9, lines 366-375

Reviewer B

This manuscript by Xu et al., assesses LUSC in the context of the expression of fatty acid metabolism genes. Based on their analysis, the authors provide a fatty acid metabolism related gene signature that they indicate can predict prognosis in early-stage LUSC. While the topic, general approach, and results are certainly suitable for publication in this particular journal, the authors need to significantly expand the information provided in this manuscript before it meets the minimum standard for publication. The authors are encouraged to address the specific points below, as this reviewer believes that the authors can achieve a publishable manuscript.

1. The materials and methods section needs to be re-written to comprehensively explain the steps the authors took to generate and analyze their data. For example, the authors need to explain how they curated the KEGG database, provide a summary explanation of the GSVA and the ESTIMATE algorithm, explain how they performed the drug response in GDSC. Currently, the authors simply state that they used these resources. They must explain how they used them and summarize how the resources curated and analyzed the data.

Reply: We thank the reviewer for the suggestion. According to the request, we re-written the **Methods** section to comprehensively explain the data collection, bioinformatics analysis, and machine learning methods. In summary, our comprehensive approach encompassed meticulous data collection, sophisticated bioinformatics analyses, and machine learning strategies to construct and validate a robust

FAMRGs-based signature for prognosis in early-stage LUSC. Additionally, our study delved into the potential clinical implications of this signature in guiding personalized treatment strategies.

Changes in the text: see Page 3-4, lines 87-158

2. The results section requires similar expansion. For example, the only reference to Figure 1 is a minimal sentence with no context. Please provide sufficient context for each figure for readers to understand what you did and why you did it. This holds true for the results text related to all figures.

Reply: As request, we added description of **Results** and **figure legends** (see the **revised supplementary file**) in detail. For example, **Figure 1** showed the flowchart of study design for FAMRGs signature in early-stage LUSC. We add the description of figure legend: A TCGA-LUSC dataset (training cohort) and six GEO-LUSC datasets (validation cohorts, including GSE73403, GSE74777, GSE50081, GSE37745, GSE30219, and GSE7157010) were included in this study. The training cohort, comprising 396 patients, was used for feature selection and LASSO-Cox regression model construction. Six independent validation cohorts, with a total of 543 patients, were assigned to confirm the model's performance.

Changes in the text: see Page 5-7, lines 166-290

3. The introduction section is unacceptable as it provides less that minimal information for readers to understand the context of this study. The authors need to significantly expand both the LUSC and fatty acid metabolism subsections of the introduction. Without an appropriate expansion of the introduction, reviewers will not be able to judge whether the authors themselves sufficiently understand the context in order to perform and accurately assess this study.

Reply: We thank the reviewer for the advice. We added more content about the LUSC and fatty acid metabolism and explain the reason why we explore the role of fatty acids metabolism-related genes (FAMRGs) in early-stage lung squamous cell carcinoma (LUSC) in the **Introduction**. Previous research has demonstrated that alterations in fatty acids metabolism and aberrant expression of fatty acids metabolism-related genes (FAMRGs) closely correlate with tumor survival and treatment resistance. For example, oxoglutarate dehydrogenase-like (OGDHL) was found to be significantly downregulated in clear cell renal cell carcinoma. The upregulation of OGDHL expression effectively inhibited cancer growth and metastasis both in vitro and in vivo. Shifeng Yang et al. showed that RGS2 was highly expressed in gastric cancer and may contribute to immune rejection, which was closely associated with a poor prognosis. Furthermore, a study by Li LY et al. demonstrated that sterol regulatory element binding transcription factor 1 (SREBF1) as a central mediator linking tumor protein 63 (TP63) with fatty acids metabolism, which regulates the biosynthesis of fatty acids, was essential for viability and migration in squamous cell carcinomas (SCC), and its overexpression was associated with poor survival in SCC patients. However, the characteristics of fatty acids metabolism alteration and its potential as a biomarker for cancer prognosis and treatment response in early-stage LUSC require further exploration. In light of this, our study employs a

bioinformatic approach to establish a robust gene signature rooted in fatty acids metabolism for prognostication.

Changes in the text: see Page 2-3, lines 68-85