Peer Review File

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Reviewer A

The paper titled "Transcription factor-target gene regulatory network analysis in human lung adenocarcinoma" is interesting. These findings revealed a novel TF-target gene regulatory axis related to tumorigenesis and the tumor immune microenvironment and provided potential therapeutic targets and mechanisms for LUAD. However, there are several minor issues that if addressed would significantly improve the manuscript.
Response: Thank you for your positive comments.

1) There have been many studies on lung adenocarcinoma. What is the difference between this study and previous studies? What is the innovation? These need to be described in the introduction.
Response: High-throughput sequencing technology has assisted in gaining a deeper understanding of the pathogenesis of LUAD and identifying multiple TFs involved in its occurrence and development. A recent transcriptional regulatory network study identified key TFs involved in lung adenocarcinoma development, including SOX10, SPIB, NR4A2, FOXD1, ELF5, HOXA5, KLF5, ESRRA, SREBF1 and REL. However, due to the diversity and heterogeneity of LUAD, our knowledge of the TF regulatory network in LUAD remains limited, particularly in terms of downstream target gene regulation networks and signaling pathways. Herein, we used transcriptomic sequencing to construct a regulatory network of key TFs and target genes involved in LUAD. Two potential key TFs involved in the regulation of LUAD (GRHL3 and TAL1) were identified, along with five alternative splicing events (XMIR, XIR, XAE, MIR, and IR) that exhibited abnormal occurrence in LUAD. We propose the existence of a GRHL3-CDH15-Wnt-β-catenin pro-oncogenic signaling axis and a TAL1-ADAMTS1-vascular antioncogenic signaling axis. We have revised the manuscript (see page 4, line 109).

2) The abstract is not enough, and the research methods is too simple. Further modifications are needed.
Response: Thank you for your kind suggestion. We have revised the abstract (see page 3, line 56).

3) How to understand the tumor immune microenvironment of lung adenocarcinoma? How to reduce the probability of recurrence after surgery? It is recommended to add related descriptions.

Response: Thank you for your constructive comments. The tumor immune microenvironment is the main source of heterogeneity in lung cancer, affecting disease progression and response to treatment. Therefore, understanding the immune microenvironment of the lung will help to better understand the mechanisms of disease progression, identify biomarkers that respond to existing therapies, and provide key insights into pre-invasive and early invasive lung adenocarcinoma (PMID: 31446140). We have added corresponding information (see page16, line 488).

4) Some gene names in Figure 3B are unclear. Please upload clearer figure again.
Response: Thank you for your kind suggestion. We have revised the figure.

5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "Systemic immune microenvironment and regulatory network analysis in patients with lung adenocarcinoma, PMID: 35116596". It is recommended to quote this article.
Response: Thank you for your kind suggestion. We have added the reference (see page 3, line 72).

6) How to screen new diagnostic and prognostic markers for lung adenocarcinoma? It is suggested to add relevant contents in the discussion.
Response: Thank you for your kind suggestion. A study using single-cell RNA sequencing revealed significant heterogeneity in early LUAD carrying EGFR mutations, suggesting complex interactions between tumour cells, stromal cells and immune infiltration in the tumour microenvironment (PMID: 33144684). Multi-region whole exome sequencing analysis revealed that mutations in EGFR, ERBB2, NRAS, and BRAF are early clonal genomic events in lung adenocarcinoma carcinogenesis, while TP53 and genes related to cell migration, gap junctions, and metastasis may be late events associated with subclonal diversity and tumor progression (PMID: 31446140). Another study established a novel lipid metabolism-based 12-gene signature for predicting recurrence, which may provide important guidance for achieving optimal anti-tumour effects in early LUAD immunotherapy or chemotherapy (PMID:35222371). A latest study utilized a highly reusable imaging quality control cytometer (IMC) to analyze the spatial resolution features related to lung tumour immune microenvironment and clinical outcomes in LUAD patients. The results showed that AI based systems can extract features from raw IMC images to predict various clinical outcomes (PMID: 36725934). We have added corresponding information (see page16, line 488).

7) What are the effects of genes and immune microenvironment on the evolution of

early lung adenocarcinoma? It is recommended to add relevant content.

Response: Thank you for your kind suggestion. Early detection is important for improving outcomes for LUAD. Understanding the immune microenvironment of the lung will help to better understand the mechanisms of disease progression and provide key insights into pre-invasive and early invasive lung adenocarcinoma. A study using single-cell RNA sequencing revealed significant heterogeneity in early LUAD carrying EGFR mutations, suggesting complex interactions between tumour cells, stromal cells and immune infiltration in the tumour microenvironment (PMID: 33144684). Multi-region whole exome sequencing analysis revealed that mutations in EGFR, ERBB2, NRAS, and BRAF are early clonal genomic events in lung adenocarcinoma carcinogenesis, while TP53 and genes related to cell migration, gap junctions, and metastasis may be late events associated with subclonal diversity and tumor progression (PMID: 31446140). We have added corresponding information (see page16, line 488).

Reviewer B

The authors of this manuscript made attempts to clarify transcription factor (TF) regulatory network in lung adenocarcinoma. Using four tissue samples and based largely on in-silico databases, they extracted 5 up-regulated and 5 down-regulated TF genes in lung cancer tissues as compared with normal lung tissues. The topic itself is interesting and must become an important area in the future, but this reviewer would like to point out the following issues to be addressed.

Response: Thank you for your positive comments.

1. “paracancer” is an immature word.

Response: Thank you for your kind suggestion. We have corrected (see page 6, line 140).

2. Is “stored at -80C in liquid nitrogen” correct? If stored in liquid nitrogen, it should be -196C.

Response: Thank you for your comment. We have corrected (see page 7, line 151).

3. How many lung cancers were used? Four cases? Please demonstrate detailed clinicopathological data of the patients, including driver mutations, histological subtypes, smoking status and TTF-1 expression by IHC (TRU-type or not).

Response: Four case are involved in the study. We have added corresponding information in the new table (see Page27, line 736).

Table 1 The patients' clinical information

Patient	Age	Gender	Side	T	N	M
No.1	73	male	LL	T1	N0	M0
No.2	71	male	RL	T1	N0	M0
No.3	79	male	LL	T1	N0	M0
No.4	68	male	RL	T1	N0	M0

4. It was stated that "in this network, among the top 5 upregulated and downregulated TFs, one TF in each group was not predicted to have consistent expression with its target genes, while the expression trends of the remaining five TFs were consistent with their corresponding target genes (Figure 3A,3B)." Are the TFs in the upregulated and down-regulated groups ETV4 and SOX17, respectively? Is "the remaining five TFS" correct? Remaining four TFs?

Response: Thanks a lot for your comment. We have corrected (see Page 10, line 260).

5. Although ETV4 and SOX17 were significantly differentially expressed, why weren't their expressions consistent with the target's genes? Please discuss.

Response: Thank you for your constructive comments. Here, we found ETV4 significantly upregulated in LUAD tissues, while SOX17 were significantly downregulated. However, the expression of ETV4 and SOX17 were not consistent with the targets genes. Meanwhile, we found ETV4 did not has significant associated on the survival rate of patients with LUAD. As functional enrichment analysis revealed that differentially expressed genes in LUAD were significantly enriched in the Wnt signaling pathway and vascular regulation pathway. Next, during the exploration of target genes regulated by key TFs, we discovered a potential oncogenic TF (GRHL3) that may regulate the Wnt signaling pathway, as well as a potential tumor-suppressive TF (TAL1) that may regulate signaling pathways associated with angiogenesis.

6. It seems that GRHL3 is related to squamous cell carcinoma, not adenocarcinoma, in the literature. How do GRHL3 and CDH15 work for lung adenocarcinoma? Myogenic differentiation has nothing to do with adenocarcinoma development.

Response: Thank you for your kind suggestion. In this study, we observed upregulated expression of GRHL3 in LUAD and found a negative correlation between GRHL3 expression and the survival rate of patients with lung cancer. Through target gene prediction and analysis using multiple databases, the results indicated CDH15, also known as M-cadherin, as a potential target gene of GRHL3. Existing research indicates that CDH15 may be associated with the occurrence, progression, and prognosis of

certain tumors. In LUAD, the Wnt signaling pathway is frequently aberrantly activated, leading to the enhanced proliferation and invasive capabilities of LUAD cells, and is involved in the EMT process and the maintenance and proliferation of LUAD stem cells. M-cadherin-mediated signaling pathway attenuates the phosphorylation of β-catenin (Ser31/37/Thr41) by GSK-3β. Therefore, we hypothesize that GRHL3 exerts its oncogenic function by transcriptionally regulating CDH15 expression and modulating the Wnt/β-catenin signaling pathway.

7. I was not able to follow the authors' logic that, although there were many signal pathways in Fig. 1C and 1D, how the Wnt pathway was extracted. Please clarify.
Response: Thank you for your comments. We found that different expression genes were enriched for molecular functions and signalling pathways, both related to Wnt. In LUAD, the Wnt signaling pathway is frequently aberrantly activated, leading to the enhanced proliferation and invasive capabilities of LUAD cells, and is involved in the EMT process and the maintenance and proliferation of LUAD stem cells. Therefore, we focused on the Wnt signalling pathway in the subsequent transcription factor analysis.

8. The readers may not understand why ADAMTS1 was extracted from the 23 genes.
Response: Thank you for your comments. Among 23 potential targets, ADAMTS1 was significantly up-regulated in LUAD tissues. It has been reported to be closely associated with angiogenesis and vascular function and to have inhibitory effects on some tumours. JASPAR database analysis identified three potential TAL1 binding sites in the ADAMTS1 promoter sequence (Figure 6). Based on the results of the above analysis, we hypothesise that TAL1 may regulate the vascular system of LUAD cells through the transcriptional regulation of ADAMTS1, thereby inhibiting the development and progression of LUAD.

9. Regarding alternative splicing: were the results shown in Fig. 7B obtained from comparisons of alternative splicing between NM (n=4) and LC (n=4)? More detailed explanation is needed.
Response: Thank you for your kind suggestion. The results shown in Fig. 7B were obtained from comparisons of alternative splicing between NM and LC. We have made corresponding revision in the results (see Page 13, line 360).

10. The first paragraph of Discussion is on Wnt signaling and angiogenesis, which is, however, not the main topic of this paper. As long as normal cancer scientists read this manuscript, they understand the paper is on the transcription factor network in lung adenocarcinoma. The first paragraph of Discussion should be drastically revised

appropriately, or even deleted.

Response: Thank you for your constructive suggestion. We have changed the first paragraph into “As a malignant tumor, LUAD’s occurrence and development are regulated by various complex molecular mechanisms. TFs play a crucial role in LUAD by regulating multiple key genes and signaling pathways, influencing the occurrence, development, and transformation of LUAD. However, the regulatory mechanisms of TFs in LUAD are highly complex. Despite previous studies identifying several important TFs in LUAD, many key TFs remain unexplored, and no research has been conducted on constructing TF-target gene regulatory networks based on the differentially expressed genes in LUAD. Therefore, in this study, we further identified key TFs in LUAD through high-throughput sequencing and, for the first time, constructed a key TF-target gene regulatory network based on the differentially expressed genes in LUAD. Interestingly, during the exploration of target genes regulated by key TFs, we discovered a potential oncogenic TF (GRHL3) that may regulate the Wnt signaling pathway, as well as a potential tumor-suppressive TF (TAL1) that may regulate signaling pathways associated with angiogenesis” (see Page 13, line 367).