Peer Review File

Article information: https://dx.doi.org/10.21037/jtd-23-317

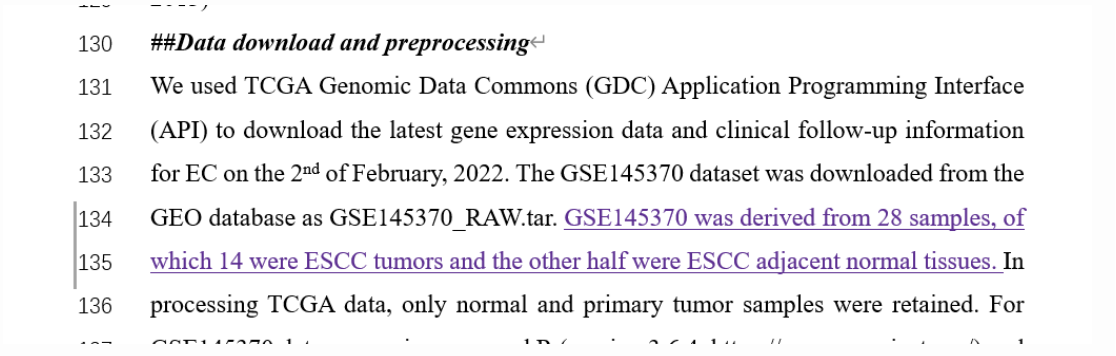
Reviewer A

In the paper titled, "Immune characteristics and genetic markers of esophageal cancer by single-cell3 analysis: implications for immunotherapy," Xu et. al. perform an analysis of existing single cell datasets to identify cellular changes within tumors and their surrounding microenvironments. This is done by deconvolution of single cell data to identify cell types and differential expression between tumor and surrounding adjacent tissue. This is a very detailed analysis of existing data and I commend the authors for their work. I believe the authors struggle, however, in understand the meaning of the data and their analysis and figures are hard to follow. In addition, I have the following comments:

1. In the methods section please clarify the number of sample analyzed and the histology of the specimens analyzed (squamous vs adenocarcinoma)

Reply: We've added this information in manuscript. GSE145370 was derived from 28 samples, of which 14 were ESCC tumors and the other half were ESCC adjacent normal tissues.

Changes in the text:



130 ***##Data download and preprocessing***

131 We used TCGA Genomic Data Commons (GDC) Application Programming Interface

132 (API) to download the latest gene expression data and clinical follow-up information

133 for EC on the 2nd of February, 2022. The GSE145370 dataset was downloaded from the

134 GEO database as GSE145370_RAW.tar. GSE145370 was derived from 28 samples, of

135 which 14 were ESCC tumors and the other half were ESCC adjacent normal tissues. In

136 processing TCGA data, only normal and primary tumor samples were retained. For

2. The results section is very difficult to follow. There are so many regions which are being discussed that it is very hard to follow in a clear and concise fashion. Using acronyms or other shorthand text may help the reader follow the results. For example, in the third paragraph, there is mention of cancer, cancer-side and then overall sample. A figure demonstrating what you are referring to as cancer and cancer-side would be helpful.

Reply: Thanks for the suggestion, we really should make the article more concise and understandable. Incorporating diagrams when reading can be easy to understand.

3. The authors should consider adding histograms to figures 2D and 5B because even tumors have significant heterogeneity and a histogram would allow the reader to see the heterogeneity of samples within conditions.

Reply: Thank you for your valuable input, tumor heterogeneity is indeed an important factor. In subsequent work, we will conduct a more in-depth analysis of the results covered in this

paper, taking into account the analysis of tumor heterogeneity, which will be presented in later results.

4. The pathway analysis figures need to be condensed to make those easier to follow. Although you list the most commonly expressed pathways, not all those need to be listed because many of those do not have impact on cancer or normal tissue biology in the esophagus.
Reply: Thanks for the advice, indeed many pathways do not seem to be related to tumors at the moment. We just presented it as it was so that we could inspire future research.

5. A patient characteristic table is needed for this paper. It is difficult to interpret clinical characteristics, staging, etc without a demographic table to understand the cohort of patients being studied. Also were all of these patients untreated prior to collection of tissue or did some of the patients undergo neoadjuvant therapy prior to tissue collection?
Reply: Patient characterization information, including staging and treatment history, is available in the GSE145370 dataset. In this paper, there is really not much ink on the analysis of the relationship between clinical features and molecular biological features, which is also the focus of our follow-up research.

6. The authors dedicate a large portion of the discussion on therapeutic implications of their findings however there really is not link between their findings and any impact on therapy. Their discussion is mostly work that has been previously done and is not directly relevant to their findings. I think a much more focused evaluation of their work would make for a better discussion and one that is easier to read.
Reply: In this article, we have struggled to present some facts to derive from the data. Our vision is that single-cell analysis of esophageal cancer samples can shed light on clinical work, which requires enormous work to achieve. This is also the focus of our future work.

Reviewer B

In this study, through bioinformatics analysis of cancer and paracancerous data of EC patients in multiple databases, differential cell subsets were screened out and molecular targets with prognostic significance were selected. Subsequent functional experiments can be performed on CXCL8.
Reply: Thank you for your valuable advice, which we are intending to do.

Reviewer C

First, the title needs to indicate what the “implications” is, i.e., prognostic roles? and the research design of this study, i.e., a bioinformatics analysis.
Reply: Thank you very much for your advice. These are described in the summary as well as in Highlight.

Second, the abstract needs to be further edited. The background did not describe what has been known on this research focus and what the knowledge gap is. The methods need to describe the variables in the databases which were used in this study, in particular the prognosis outcomes and clinical factors. Please specify the methods used for the bioinformatics analysis and the ascertainment of the prognostic roles of the biomarkers. The results need to quantify the findings by reporting outcome values and P values. The conclusion needs to be more detailed for the comments for the clinical implications of the findings.

Reply: First of all, thank you very much for your advice. In this article, we have struggled to present some facts to derive from the data. Our vision is that single-cell analysis of esophageal cancer samples can shed light on clinical work, which requires enormous work to achieve. We hope to use more data to build and validate a relevant model that can be used to predict prognosis. This is also the focus of our future work.

Third, in the introduction of the main text, the authors need to review the findings from traditional RNA sequencing studies on the immune characteristics and genetic markers. Please also analyze the limitations and knowledge gaps of prior studies on this research focus. The authors need to explain why the bioinformatics analysis is suitable to address this research question.

Reply: Thank you for your suggestion. Relevant content has been added in the text.

Changes in the text:

more in-depth study of the transcription group characteristics of EC and a deeper understanding of the origin and development of EC. RNA-seq provides an effective high-throughput technique for reliably characterizing tumor immune microenvironments. Based on this technique, the key components of immunoinfiltration, expression phenotype and immune pool in anti-tumor immune response can be revealed[19].However, studies based on traditional RNA sequencing have focused on the overall level of the sample, and more research is needed to understand the heterogeneity of a cluster of cells and individual cells. Single-cell

Fourth, in the methodology of the main text, please consider to use a flowchart to describe the methodology details of this study. The authors need to describe the dataset used including the variables and outcomes in them. The authors need to use a separated paragraph to describe the statistical methods including how the prognostic roles were ascertained.

Reply: Thank you for your suggestions, which are described in the main text. In addition, prognostic correlation analysis and research will be involved in future work, please look forward to it.

Reviewer D

1. Please indicate the full name of "ESCC" below. Abbreviated terms should be full when they first appear.

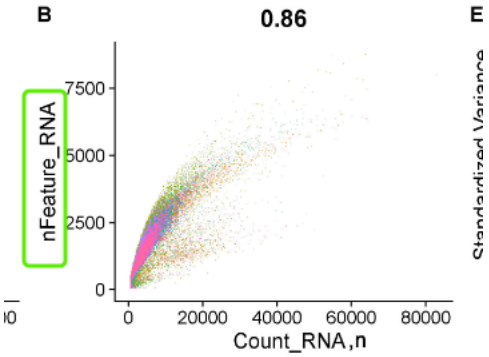
93 interaction with pericyte cells [11].At the same time, it has been reported that some
94 immune-related parameters can be used to predict the prognosis of ESCC patients([12,
95 13] [14, 15]), which also suggests that these immune parameters have a profound

Reply: We have made changes in the manuscript.
Changes in the text:

93 interaction with pericyte cells [11].At the same time, it has been reported that some
94 immune-related parameters can be used to predict the prognosis of ESCC (Esophageal
95 Squamous Cell Carcinoma) patients([12, 13] [14, 15]), which also suggests that these

2. There are two reference lists in your manuscript. Please check.
Reply: We have made changes in the manuscript.

3. Figure 1:
Please revise “nFeature-RNA” to “Feature-RNA, n” in Figure 1B.



Reply: We have made changes in the Figure 1B.