Peer Review File
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Reviewer A

Comment 1: Did the study only look at the limbs of the limbs? Other places will be fun, too.

Reply 1: Thank you for your kind comment. In our study, we acquired four fresh samples for single-cell RNA sequencing, with two originating from the pelvic area to the right buttock, one situated within the abdominal and pelvic cavity, and one located posterior to the right eyeball. Recognizing the rarity of infantile fibrosarcoma (IFS), we consider ourselves fortunate to have procured lesion tissues from diverse anatomical sites, allowing for a comprehensive depiction of IFS at the single-cell level. Additionally, the remaining samples utilized for immunohistochemistry encompassed various anatomical sites including limbs, head, and trunk. To maintain conciseness in the manuscript, we have delineated the clinical features in Table 1 and Table 2 (see Page 21, line 677-682 and the attachment "Tables"). Also, wo have modified the inappropriate text in the Abstract (see Page 2, line 39-40) and Introduction section (see Page 3, line 69-71, 76-79) to avoid misunderstanding.

Changes in the text:

'Its primary therapeutic intervention places patients at a risk of disability or mutilation.'

'It is the most common soft tissue sarcoma in children under 1 year old, with a higher incidence in males than in females, and is often located in the distal extremities (1).'

'This significantly complicates the possibility of radical surgical resection, which is the primary therapeutic intervention for IFS, and places the patient at a considerable risk of disability or mutilation.'

Reviewer B

Thank you for the opportunity to read your paper. However, I find it unsuitable for publication and lacking of foundation.

Comment 1: First, diagnosis of cannonical infantile fibrosarcoma is usually non-problematic, panTrk immunohistochemistry might be of aid in less morphologically typical cases, and virtually all cases can be resolved with molecular-genetic methods. You do not mention anywhere in the text that there is an emmerging group of infantile fibrosarcoma-like tumors with other kinase genes abberations, with similar biologic behaviour and methylation profiles concordant with infantile fibrosarcoma - these are usually also easily diagnosed with targeted RNA-seq. Molecular-genetic methods are

necessary as they might make the patient eligible for targeted therapies if needed. I mean targeted therapies that are already FDA approved and proved effective in some infantile fibrosarcoma cases.

Reply 1: Thank you for the important comment. We have added the data of infantile fibrosarcoma-like tumors with other *NTKR* gene-unrelated abberations in the Introduction section (see Page 3, line 87-91). Also, we performed the scRNA-seq to seek novel and more specific markers for the immunohistochemical diagnosis and treatment of IFS at a single level and had no objections to molecular-genetic methods.

Changes in the text: 'However, *NTRK* gene-related chromosomal translocations have been confirmed to be be relevant to other tumors, and other *NTRK* gene-unrelated chromosomal variations, such as *BRAF* rearrangements, have been reported in IFS (1, 5). This suggests that this diagnostic approach, despite its high cost, may lack specificity and universality to some extent.'

Comment 2: Second, even though the findings might be of some value, I am not sure you bring anything groundbreaking to the table. Neoangiogenesis and immune system response are features that are seen in any tumor in general. I must also question the sensitivity of your methods since you only found cancers stem cells in 3/4 cases. Why weren't they found in the fourth case? What sustains the tumor growth in this case?

Reply 2: Our study represents the first single-cell RNA sequencing (scRNA-seq) investigation of IFS, hopefully providing a novel perspective on tumor heterogeneity and the tumor microenvironment. In the manuscript, we designate diverse malignant cell populations as 'tumor 1', 'tumor 2', 'tumor 3', and 'tumor 4', rather than indicating their sample origins. This can be observed in the Results section (see Page 10, line 307-318).

Reviewer C

The paper titled "Characterization of the malignant cells and microenvironment of infantile fibrosarcoma via single-cell RNA sequencing" is interesting. This study provides a comprehensive characterization of the tumor transcriptome and TME of IFS at the cellular level, offering valuable insights for clinically significant advancements in the diagnosis and treatment of IFS. However, there are several minor issues that if addressed would significantly improve the manuscript.

Comment 1: This study mentioned some cell subpopulations and suggested analyzing the heterogeneity and functional changes of different cell subpopulations in IFS patients.

Reply 1: Thank you for the important comment. We have added further analysis of subgroup heterogeneity and functional changes in the Discussion section (see Page 12-13, line 403-412).

Changes in the text: ‘Moreover, we identified four malignant cell subtypes with distinct functions denoted as tumor 1, tumor 2, tumor 3, and tumor 4). According to the GO analysis, tumor 1, potentially characterized by myofibroblast-like malignant cells, could participate in facilitating tumor invasion and metastasis through its involvement in actin cytoskeleton reorganization (48). Tumor 2 could exhibit heightened proliferation and adhesion capabilities, suggesting its role as a subgroup primarily responsible for tumor growth (49). Tumor 3 could get involved in immune responses, while tumor 4 appeared to be a subgroup with a lower degree of differentiation. The latter three subgroups could collaborate in reshaping the non-cellular components of the tumor microenvironment, thereby fostering the progression of IFS (50).’

Comment 2: The abstract is not sufficient and needs further modification. The research background did not indicate the clinical needs of the research focus.

Reply 2: Thank you for the kind and precise comment. We agree with your comment and have modified our text as advised in the Abstract section (see Page 2, line 37-42) and in the Introduction section (see Page 3, line 87-91; Page 4, line 112-115) for highlighting the clinical needs of the research focus.

Changes in the text:

‘The tumor lacks specific immunohistochemical tumor marker and a general view of tumor microenvironment (TME). Its primary therapeutic intervention places patients at a risk of disability or mutilation. This study aimed to elucidate the universal transcriptional characteristics of IFS and explore novel targets for diagnosis and therapy using single-cell RNA sequencing (scRNA-seq).’

‘However, NTRK gene-related chromosomal translocations have been confirmed to be be relevant to other tumors, and other NTRK gene-unrelated chromosomal variations, such as BRAF rearrangements, have been reported in IFS (1, 5). This suggests that this diagnostic approach, despite its high cost, may lack specificity and universality to some extent. Furthermore, the related targeted therapies also face certain challenges, including tumor resistance, adverse reactions, and high costs (8).’

‘We therefore conducted scRNA-seq on lesion tissues from patients with IFS to reveal the universal transcriptional characteristics of both the malignant cells and the TME within this disease, and identify novel targets for immunohistochemical diagnosis, which demands lower cost, and treatment.’

Comment 3: This study is based on bioinformatics analysis. It is recommended to increase in vivo and in vitro experimental studies, which may be more meaningful.

Reply 3: Thank you for the constructive suggestion. Validating bioinformatics analysis results through in vitro and in vivo studies, and obtaining more meaningful outcomes has been a primary focus of our research efforts. However, we are currently constrained

by the unavailability of established IFS models. Nevertheless, we are actively endeavoring to establish patient-derived cell and patient-derived xenograft models for subsequent investigations. If there are any advancements, we will promptly compile them into a manuscript for publication.

Comment 4: That is the value of single-cell RNA sequencing technology in exploring tumor heterogeneity? What is the biggest challenge facing? It is suggested to add relevant contents.

Reply 4: Thank you for the helpful suggestion. We have added the value of single-cell RNA sequencing technology in exploring tumor heterogeneity in the Introduction section (see Page 3-4, line 102-111) and the biggest challenge of this technique in the Discussion section (see Page 14, line 458-461).

Changes in the text:

'In recent years, single-cell RNA sequencing (scRNA-seq) has emerged as a robust analytical technique that allows for the investigation of omics information at the individual cell level (12). It provides unprecedented resolution for comprehensively understanding the cellular diversity within complex cancers. Through comparative analysis with normal cells, scRNA-seq can discern malignant cells, characterize distinct malignant cell populations, and elucidate their shared or unique gene expression profiles and functions (13). This advancement holds promise for prognostic prediction and targeted therapy development. Furthermore, scRNA-seq facilitates the investigation of the TME and the interactions among its cellular constituents, providing valuable insights for targeted immunotherapy (14).'

'Second, single-cell sequencing analysis is conducted based on bioinformatics methods and is limited to the mRNA level, requiring further experimental support and integration with multi-omics analysis to characterize tumor heterogeneity comprehensively. '

Comment 5: In the process of tumor occurrence and development, the tumor microenvironment plays a vital role. Please briefly introduce the research progress of tumor microenvironment heterogeneity in IFS.

Reply 5: Thank you for the professional suggestion. We have add the research progress of tumor microenvironment heterogeneity of IFS in the Introduction section (see Page 3, line 93-101) and modified our text as advised in the Discussion section (see Page 12, line 375-380).

Changes in the text:

'The tumor microenvironment (TME), which directly influences cancer cells and plays a crucial role in cancer development and progression, has become a focal point of breakthrough research in emerging therapeutic strategies. Regarding IFS, ZHU et al.

have utilized immunohistochemistry and multicolor flow cytometry to prove that IFS tumors are highly immunogenic (11). They propose that the expansion of tumor-infiltrating lymphocytes followed by adoptive cell transfer could be a potential immunotherapy for IFS patients. Yet, the overall and precise characteristics of the TME, as well as the interactions between malignant cells and other cells within IFS, remain largely unknown.'

'TME, recently, which directly influences cancer cells and plays a crucial role in cancer development and progression, has emerged as a rapidly expanding area of interest. A better understanding of the structural and the functional characteristics of TME provides insights into potential novel therapeutic targets, including immunotherapies and anti-angiogenic therapies (41). However, the comprehensive characteristics of the TME within IFS is unclear.'

Comment 6: The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "Bioinformatics analysis and single-cell RNA sequencing: elucidating the ubiquitination pathways and key enzymes in lung adenocarcinoma, PMID: 37559628". It is recommended to quote the article.

Reply 6: Thank you for the kind and precise suggestion. We have modified the text as advised and added this article quotation in the in the Introduction section (see Page 3-4, line 102-111).

Changes in the text:

'In recent years, single-cell RNA sequencing (scRNA-seq) has emerged as a robust analytical technique that allows for the investigation of omics information at the individual cell level (12). It provides unprecedented resolution for comprehensively understanding the cellular diversity within complex cancers. Through comparative analysis with normal cells, scRNA-seq can discern malignant cells, characterize distinct malignant cell populations, and elucidate their shared or unique gene expression profiles and functions (13). This advancement holds promise for prognostic prediction and targeted therapy development. Furthermore, scRNA-seq facilitates the investigation of the TME and the interactions among its cellular constituents, providing valuable insights for targeted immunotherapy (14).'

'13. Lu T, Xu R, Wang C, Zhou X, Parra-Medina R, Díaz-Peña R, et al. Bioinformatics analysis and single-cell RNA sequencing: elucidating the ubiquitination pathways and key enzymes in lung adenocarcinoma. J Thorac Dis. 2023;15(7):3885-907.'

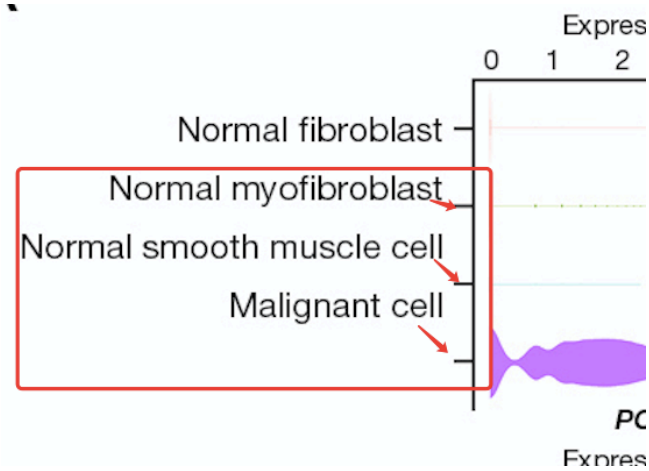
Comment 7: How to use single-cell RNA sequencing technology to screen new diagnostic and prognostic markers for IFS? It is suggested to add relevant contents.

Reply 7: Thank you for the constructive suggestion. We have modified our text as advised in the Results section (see Page 9, line 291-297) to make the methods to screen new diagnostic and prognostic markers for IFS more detailed and comprehensive.
Changes in the text: 'To identify potential novel markers or therapeutic targets for IFS, we conducted differential gene expression analysis by comparing the total malignant cells of four samples with normal cells from the public database. In all, we identified 1,249 DEGs. Among them, we identified three protein-coding genes (POSTN, IGFBP2, and CTHRC1) as potential novel markers for IFS, which were expressed in over 50% of malignant cells and exhibited an average fold change >1.5 and delta percentage >90% compared to normal cells (Figure 2A) (19).'

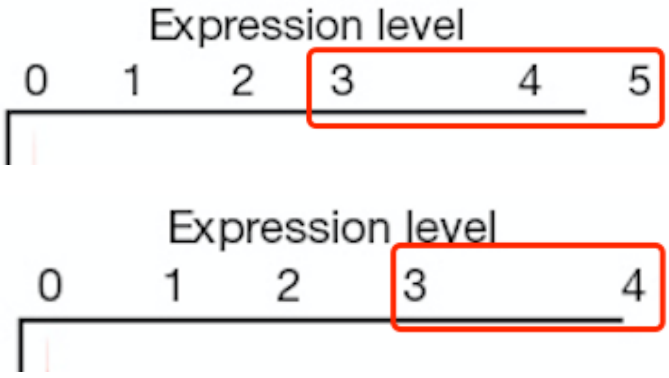
Reviewer D

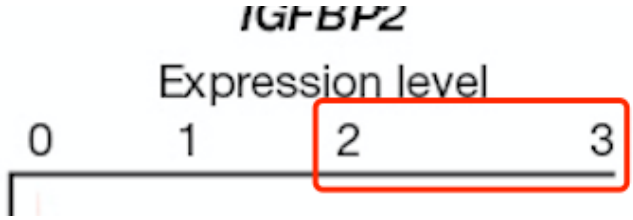
Figure 2

1. The cells are not parallel with the bar. Please revise.



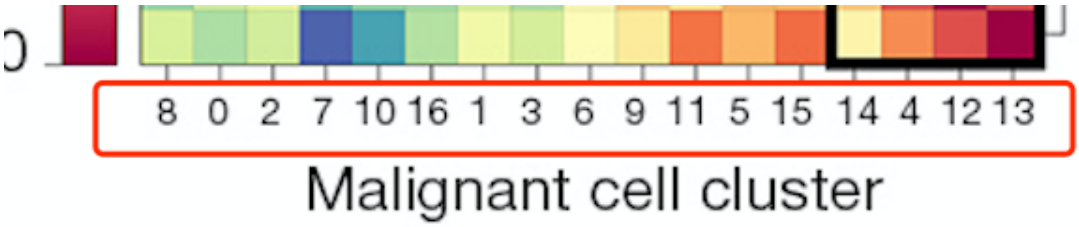
2. The spaces are not the same while these numbers are regularly arranged. Please check and revise.





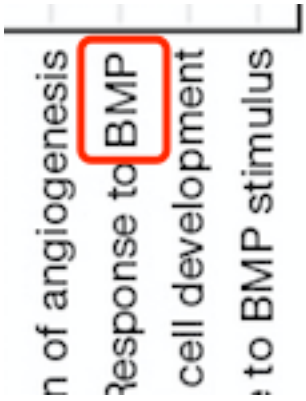
Reply: Thank you for the constructive suggestion. We have modified the Figure2 as advised (see Page 22, line 684 and the attachments).

3. These numbers are not in order regularly. Please confirm if they are correct.



Reply: Thank you for the kind suggestion. These numbers correspond to different malignant cell clusters and are ranked according to the Pearson correlation, which has been confirmed.

4. Please indicate the full term of ‘in figure legend.



Reply: Thank you for the important suggestion. We have modified the text as advised (see Page 22, line 696-697).
Changes in the text: ‘BMP, bone morphogenetic protein’