nature portfolio

Peer Review File

Single-cell dissection of multifocal bladder cancer reveals variations between primary and relapsed tumor lesions

Corresponding Author: Professor Chenfei Wang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors provide a comprehensive analysis of the intraregional heterogeneity in NMIBC specimens. This is a significant study, as intraregional heterogeneity may impact response to current therapies and progression to MIBC. However, there are some minor concerns that need to be addressed before this study is published.

1. The authors use scRNA-seq to analyze tumor regions from NMIBC patients. They derive gene signatures to stratify survival probability in public MIBC datasets. It is recommended that the authors also assess the prognostic value of their signatures in publicly available NMIBC cohorts (e.g., https://doi.org/10.1016/j.ccell.2016.05.004) as this is more relevant to their tissue of analysis.
2. The authors analyze the fibroblast and endothelial components of the primary and relapsed tumors. However, they do not elaborate on these results in their discussion or how they correlate with other similar studies in bladder and other cancers.
3. The sample number is small, and this study would benefit from a larger cohort. However, the sampling limitations are understandable.
4. In line 414 of their manuscript, the authors mention that their study aids in the "understanding of the spatial dynamics of tumor heterogeneity." However, scRNA-seq analysis does not provide any spatial information, so this sentence needs to be revised.

Overall, this is a very important study that will benefit the scientific community by providing a better understanding of the heterogeneity of multi-regional bladder cancer.

Reviewer #2

(Remarks to the Author)
This is an interesting study that applies single cell RNAseq to dissect tumor-intrinsic and extrinsic (immune microenvironment) features of non-muscle invasive bladder cancer (NMIBC). Very few single cell studies have been published in NIMBC, so this work definitely adds novelty to the field. The authors define new malignant programs and enrichment of immune cell populations implicated in tumor recurrence.

General comments:

- Recommend to replace "relapse" for "recurrence" which is most commonly used in non-muscle invasive bladder cancer literature.

- Some figures are quite dense and hard to follow (e.g. Fig 1-4) with panels that could be re organized or moved to suppl material.

- One major drawback is the low number of cases (total of 5 patients, 10 tumors), however the number of cells and diversity of cell populations is high.

- the authors should provide clinical characteristics (gender, age, smoking status, intravesical treatments) in suppl table 1.

- I feel it's hard to draw strong conclusions regarding differences and similarities of malignant cells across patients (Fig. 1) because of potential batch effects. My recommendation would be to integrate and do batch correction (e.g. Harmony), avoid such comparisons and avoid taking strong conclusions.
Minor: Fig 1 A. has a typo on "verification".

- the biological role of the stroma and tumor recurrence/modulation of the TME (Fig. 5) seem hard to reconcile. These tumors are by definition non-invasive, so the tumor cells are on the lining of the bladder at the level of the lamina propria, without contact with the underlying stroma and muscle layer. I would suggest to keep the description of stroma cell populations as part of the single cell atlas, but avoid statements around the functional relationship between stroma cells and their contribution to tumor recurrence.

- Survival analysis/bulk cohorts -- the TCGA does not seem like the ideal cohort as the bladder TCGA tumors are from muscle-invasive bladder cancer. I would suggest to validate the clinical relevance of the gene programs in non-muscle invasive bladder cancer cohorts (e.g. UROMOL). Moreover, the extrapolation and validation of macrophage signatures in other TCGA cohorts e.g. kidney (Fig. 4) seems out of the scope of this manuscript, are not statistically very robust and distracts the flow of the paper.

- the tumor-immune interactions and results around NK/macrophage subpopulations are very interesting and quite novel in this disease context.

- Lastly, congratulate the authors for making the code available in GitHub. Please consider making the raw data publicly available to the international scientific community.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors have responded to my comments adequately and have revised the manuscript accordingly.

Reviewer #2

(Remarks to the Author)
I have no additional comments. The authors addressed all my points. My and me team are available to write an editorial about this manuscript if the Editors feel it's useful as it brings novelty to the bladder cancer field.

Single-cell dissection of multifocal bladder cancer reveals malignant and immune cells variation between primary and relapsed tumor lesions

Shenghua Liu[1,#,*], Chenchen Feng[1,#], Linyi Tan[1,#], Dengwei Zhang[4], Yong-xin Li[4], Ya Han[2,3,*], Chenfei Wang[2,3,*]

Response to Reviewer's Comments

We are grateful to the editors and reviewers for their time and effort in reviewing our manuscript. Their constructive and valuable feedback has enabled us to substantially improve this work. The major updates of the revised manuscript are summarized into the following three aspects:

Evaluating the prognostic effects of signatures in public NMIBC datasets.

- We have evaluated the prognostic value of the signatures mentioned in the original manuscript within a public NMIBC cohort comprising 460 patients (Fig. R1). The results indicate that the signature gene Macro_SPP1 and the expression of IL4I1 are consistently associated with decreased survival.

Comparison of NMIBC-derived stromal cells with MIBC and other cancer types.

- We systematically evaluated whether the gene signatures derived from pan-cancer and bladder cancer-specific stromal cells are expressed in NMIBC-derived stromal cells. The results demonstrate that the stromal cells in NMIBC consistently express these marker genes and may still be influenced by the tumor microenvironment (Fig. R2).

Presentation of the current results and language improvements.

- We have significantly improved the language of the manuscript by correcting inappropriate and misleading descriptions.
- We have reorganized the figures, moving the supportive figures to the supplemental section to enhance comprehensibility.

We provided a point-by-point response as follows. The editors' and reviewers' comments are in *blue italics*, followed by our responses in black regular font.

Assess the prognostic value of the signatures in publicly available NMIBC cohorts.

Reply: We have evaluated the prognostic value of the signatures mentioned in the original manuscript within the public NMIBC cohorts (**Cancer Cell**, 2016), which includes 460 patients with paired

expression and clinical data. Consistent with the prognostic effects observed in the TCGA bladder cancer cohort, the gene signatures of Macro_SPP1 as well as the expression of IL4I1 were associated with reduced survival. Detailed responses can be found in **Point 1 of Reviewer 1 and Point 8 of Reviewer 2.**

Provide clinical characteristics about patients.

Reply: We have provided detailed clinical information in the revised manuscript (Supplementary Table 1). The complete response can be found in **Point 4 of Reviewer 2**.

Validate the results using a different cohort of patients on TCGA.

Reply: We have reorganized the manuscript in accordance with **Point 8 of Reviewer 2**'s comments, removing the extrapolation and validation of macrophage signatures in other TCGA cohorts to enhance clarity. Additionally, we have evaluated the prognostic value of these signatures using data from the public NMIBC dataset.

Reviewer #1:

Remarks to the Author:

Authors provide a comprehensive analysis of the intraregional heterogeneity in NMIBC specimens. This is a significant study, as intraregional heterogeneity may impact response to current therapies and progression to MIBC. However, there are some minor concerns that need to be addressed before this study is published.

Reply: We sincerely appreciate the reviewer's thoughtful feedback and acknowledgment of the potential value that our analyses can contribute to therapies and the understanding of disease progression. In response to the reviewer's comments, we have validated the signature genes using a publicly available dataset for non-muscle-invasive bladder cancer (NMIBC) and demonstrated consistent results with the TCGA muscle-invasive bladder cancer (MIBC) cohorts. Furthermore, we have assessed the expression of stromal cell marker genes derived from both bladder studies and pan-cancer analyses. Our observations reveal that stromal cells in NMIBC exhibit similar gene expression profiles to those identified in pan-cancer studies. This suggests that, although stromal cells in NMIBC may not be in direct contact with malignant cells, they can still be influenced by the tumor microenvironment.

1. The authors use scRNA-seq to analyze tumor regions from NMIBC patients. They derive gene signatures to stratify survival probability in public MIBC datasets. It is recommended that the authors also assess the prognostic value of their signatures in publicly available NMIBC cohorts (e.g., https://doi.org/10.1016/j.ccell.2016.05.004) as this is more relevant to their tissue of analysis.

Reply: We greatly appreciate the reviewer's insightful suggestion. In the revised manuscript, we have incorporated an evaluation of signature genes associated with survival in the NMIBC cohort, as recommended by this reviewer[1]. This cohort comprises 460 patients with paired gene expression and clinical data. In line with their prognostic significance observed in the TCGA MIBC datasets, both Macro_SPPI signature expression and IL4I1 levels are linked to poorer survival outcomes in the NMIBC dataset (Fig. R1).

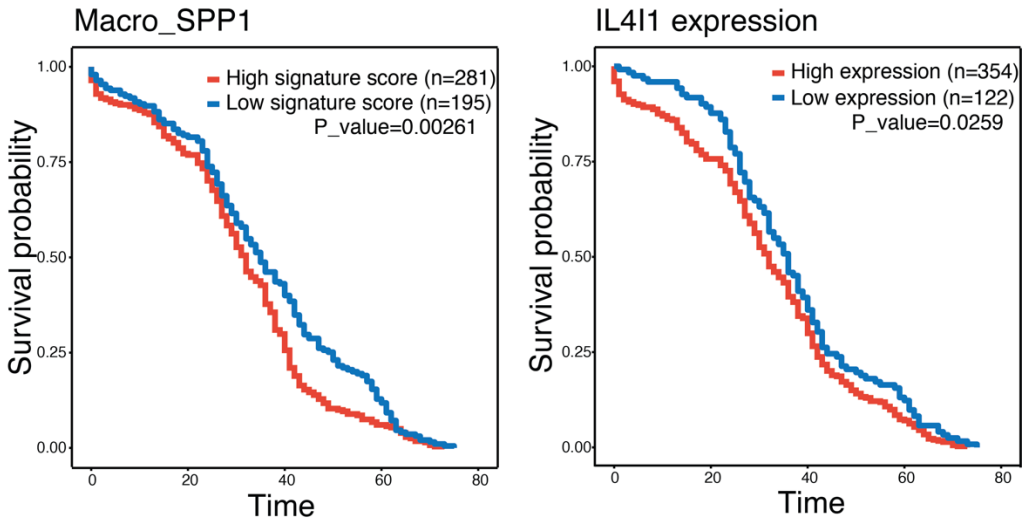

Fig. R1. Survival association in the NMIBC dataset

Kaplan-Meier curves illustrating the clinical effect of SPP1^{+} macrophages (left) and IL4I1 expression (right) in NMIBC dataset.

2. The authors analyze the fibroblast and endothelial components of the primary and relapsed tumors. However, they do not elaborate on these results in their discussion or how they correlate with other similar studies in bladder and other cancers.

Reply: We are grateful to the reviewers for their valuable suggestions. We agree with this reviewer that most current research on bladder cancer focuses on muscle-invasive bladder cancer (MIBC), while relatively little attention has been given to non-muscle invasive bladder cancer (NMIBC). Therefore, exploring the relationship of stromal cells between NMIBC and MIBC, as well as with other cancer types, allows readers to better understand the heterogeneity of stromal cells in different tumor microenvironments (TMEs).

To facilitate our comparison, we aimed to evaluate the expressed gene signatures and expression matrices for pan-cancer and MIBC-specific stromal cells, including fibroblast and endothelial cells[2,3,4]. Unfortunately, the raw scRNA-seq count matrices from two MIBC studies are not publicly available, and our attempts to obtain them were unsuccessful despite applying for access[2,3]. This limitation restricted our ability to assess the heterogeneity of cell type compositions and expression similarities between NMIBC and MIBC. In light of this, we collected pan-cancer and MIBC-specific marker genes for fibroblasts and endothelial cells[2,4,5]. We then evaluated the expression of these marker genes separately for each cell type. Our analyses revealed that nearly all marker genes for pan-cancer and MIBC-specific fibroblasts were expressed in fibroblasts derived from NMIBC (Fig.R2A). Similarly, distinct endothelial cell marker genes exhibited

comparable expression across various specific endothelial cell types (Fig. R2B). These findings suggest that the stromal cells in NMIBC are generally consistent with those in MIBC and other cancer types.

In the revised manuscript, we have incorporated the expression of marker genes in stromal cells into Supplemental Figure 5 and provided a detailed description of the relationship between stromal cells and different cancer types in the discussion section.

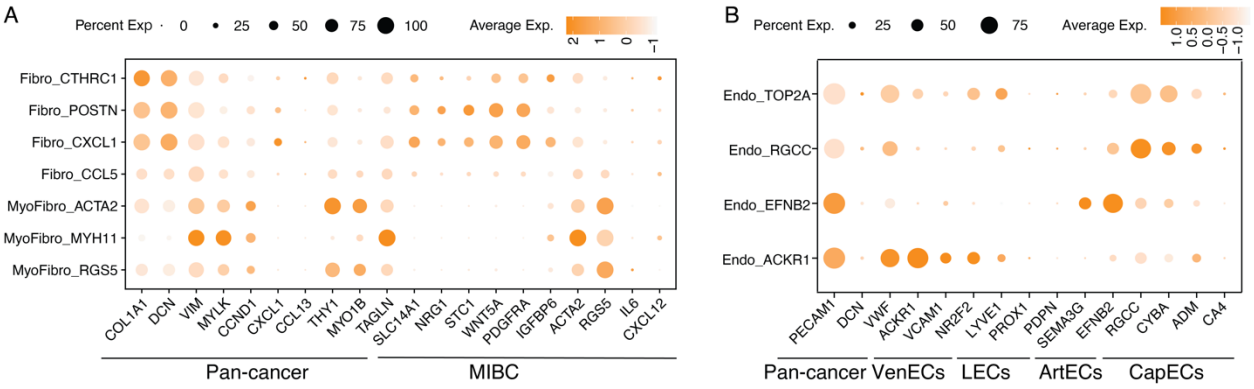

Fig. R2. Expression of markers in stromal cells

(A) Dot plot depicting the expression of pan-cancer and MIBC-specific fibroblast marker genes across various fibroblast cell types. The size and color of the dots represent the percentage of cells expressing each gene and the average expression value, respectively. (B) Dot plot depicting the expression of marker genes for venous endothelial cells (VenECs), lymphatic endothelial cells (LECs), arterial endothelial cells (ArtECs), and capillary-like endothelial cells (CapECs) across various endothelial cell types.

3. The sample number is small, and this study would benefit from a larger cohort. However, the sampling limitations are understandable.

Reply: We thank and acknowledge the reviewer for this insight. Increasing the sample size would certainly strengthen the study. As noted by the reviewer, the constraints on our sampling are understandable. To compensate for this, we were bolstering the reliability of our conclusions by validating them against public NMBIC datasets.

4. In line 414 of their manuscript, the authors mention that their study aids in the "understanding of the spatial dynamics of tumor heterogeneity." However, scRNA-seq analysis does not provide any spatial information, so this sentence needs to be revised.

Reply: We apologize for the inappropriate description. In the revised manuscript, we have corrected the sentence to focus on how our analysis provides insights into the heterogeneity of separate tumor lesions, rather than on spatial heterogeneity.

Reviewer #2:

Remarks to the Author:

This is an interesting study that applies single cell RNAseq to dissect tumor-intrinsic and extrinsic (immune microenvironment) features of non-muscle invasive bladder cancer (NMIBC). Very few single cell studies have been published in NIMBC, so this work definitely adds novelty to the field. The authors define new malignant programs and enrichment of immune cell populations implicated in tumor recurrence.

Reply: We are deeply appreciative of the reviewer's acknowledgment of our single-cell analysis for NMIBC patients. In response to the reviewer's comments, we have significantly improved the writing, relocated several figures to the supplementary materials, and applied batch correction using CCA on malignant cells. We believe these modifications have significantly improved the readability of the manuscript.

General comments:

1. Recommend to replace "relapse" for "recurrence" which is most commonly used in non-muscle invasive bladder cancer literature.

Reply: Thank you for your careful review and constructive suggestions. We have revised the manuscript accordingly.

2. Some figures are quite dense and hard to follow (e.g. Fig 1-4) with panels that could be re organized or moved to suppl material.

Reply: We would like to thank the reviewer for bringing this issue to our attention. In order to enhance the clarity and readability of the figures, we have reorganized Figures 1-4 and relocated some supporting figures—those that do not contribute to the major conclusions of this paper—to the supplementary materials.

3. One major drawback is the low number of cases (total of 5 patients, 10 tumors), however the number of cells and diversity of cell populations is high.

Reply: We fully agree that increasing the sample size would undoubtedly strengthen the study. While we recognize that our analysis is limited to 5 patients with 10 tumors, it encompasses more

than 120,000 high-quality cells, enabling us to effectively investigate heterogeneity. Additionally, to mitigate the limitations of the sample size, we validated our findings using immunofluorescence on corresponding samples and incorporated datasets from the TCGA bladder cancer study, along with a public NMIBC cohort.

4. the authors should provide clinical characteristics (gender, age, smoking status, intravesical treatments) in suppl table 1.

Reply: We have incorporated clinical information into the revised Supplemental Table 1, including gender, age, smoking status, and intravesical treatments, as per your kind suggestion.

5. I feel it's hard to draw strong conclusions regarding differences and similarities of malignant cells across patients (Fig. 1) because of potential batch effects. My recommendation would be to integrate and do batch correction (e.g. Harmony), avoid such comparisons and avoid taking strong conclusions.

Reply: We thank the reviewer for raising these important issues. We agree with the reviewer's assessment that there may be a potential batch effect in malignant cells.

To address this risk, we utilized the batch effect correction method Harmony[6], as recommended by the reviewer, alongside using canonical correlation analysis (CCA)[7], which was employed to correct the batch effect within immune cells in our previous manuscript. Compared to the original cell distribution, cells were grouped by cell types rather than by samples (Fig.R3A, corrected by Harmony, Fig. R3B, corrected by CCA), indicating that both methods effectively corrected the batch effect. However, cells derived from the P02-R1 sample remained clustered together even after Harmony correction. Then, we assess the efficacy of the two batch effect correction methods using the Local Inverse Simpson's Index (LISI)[8] and entropy scores[9,10], which offer insights into the representation of diverse datasets within local neighborhoods. Higher LISI and entropy scores indicate lower batch effects, as they reflect greater diversity within the datasets. Consistent results observed from both LISI and entropy scores suggest that the CCA method is more suitable for our malignant cells (Fig. R3C). Therefore, in the revised manuscript, we updated the associated figures and method section by CCA corrected result.

Determining whether the observed batch effect within malignant cells is due to technical factors or genuine biological variations between patients is challenging, given the unique characteristics of malignant cells, which differ from those of immune and stromal cells. Consequently, we decided to limit our analyses of malignant cells to transcription counts normalized solely by sequence depth, rather than using transcription levels corrected by CCA. We also apologize for any lack of

clarity in our explanation regarding the interregional similarity between primary and recurrent tumors; this similarity is represented by the Pearson's correlation calculated from the sequence-depth normalized transcriptomic counts based on the top 3,000 most variable genes. As a result, the findings related to cellular similarity, as depicted in the original Figures 1E-G and I, remain unchanged. Finally, in the revised manuscript, we have moderated the comparison of interregional similarity between primary and recurrent tumors.

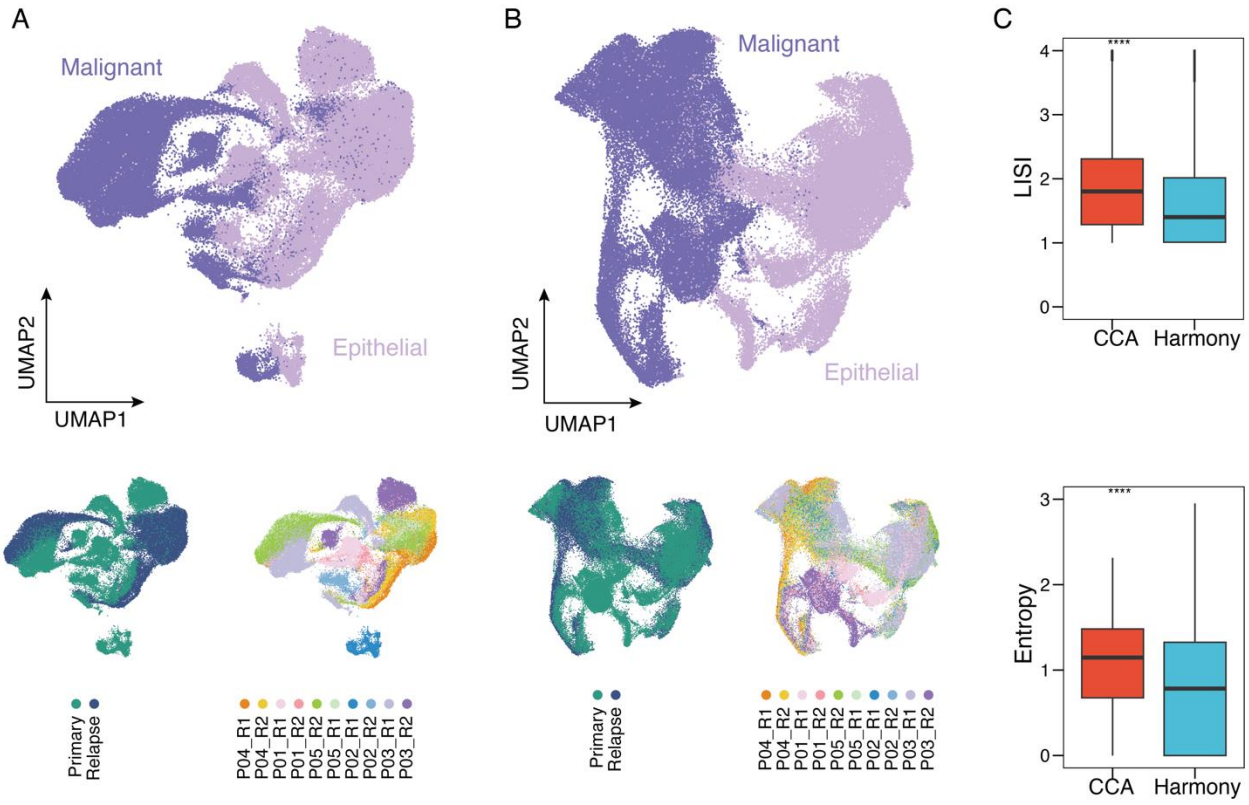

Fig.R3. Batch effect correction

(A) Uniform manifold approximation and projection (UMAP) visualization of all malignant and epithelial cells corrected by harmony, colored by cell type, source, and their patient origin respectively. (B) UMAP visualization of all malignant and epithelial cells corrected by CCA, colored by cell type, source, and their patient origin respectively. (C) Boxplot showing the distribution of LISI score and Entropies of separately corrected batch effect by harmony and CCA. P-values were computed using the two-sided Wilcoxon test.

Minor:

6. Fig 1 A. has a typo on "verification".

Reply: We are sorry for this mistake and have corrected it.

7. the biological role of the stroma and tumor recurrence/modulation of the TME (Fig. 5) seem hard to reconcile. These tumors are by definition non-invasive, so the tumor cells are on the lining of the bladder at the level of the lamina propria, without contact with the underlying stroma and muscle layer. I would suggest to keep the description of stroma cell populations as part of the single cell atlas, but avoid statements around the functional relationship between stroma cells and their contribution to tumor recurrence.

Reply: Thank you for your insightful comments. We would like to clarify that our analyses focused specifically on non-muscle invasive bladder cancer (NMIBC), not non-invasive bladder cancer. According to the staging system established by the American Joint Committee on Cancer (AJCC), NMIBC is divided into Ta and T1 stages. At the T1 stage, the tumor invades the lamina propria, which consists primarily of loose connective tissue containing numerous elastic fibers, a diverse array of stromal cells, and a few delicate, discontinuous smooth muscle bundles.

The majority of patients in our study were at the T1 stage (as shown in Supplemental Table 1), where the stromal cells derived from the tumor can interact directly with malignant cells and other cells in the TME. This context allows for significant potential interactions among stromal cells and TME cells. Although we included patients at the Ta stage, the fibroblasts derived from this stage exhibited high expression of tumor tissue-derived fibroblast marker genes (Fig. R4A). Moreover, while the endothelial cells derived from the Ta stage did not express the pan-cancer marker PECAM1, they displayed upregulation of genes associated with angiogenesis and tip cell formation, such as RGCC, which is known to correlate with poorer survival outcomes in various cancer types[10, 11] (Fig. R4B).

These findings suggest that stromal cells in NMIBC may exert mutual influences on TME cells through the secretion of cytokines. Consequently, we believe that exploring the functional relationship between stromal cells and their role in tumor recurrence is of considerable significance for this study.

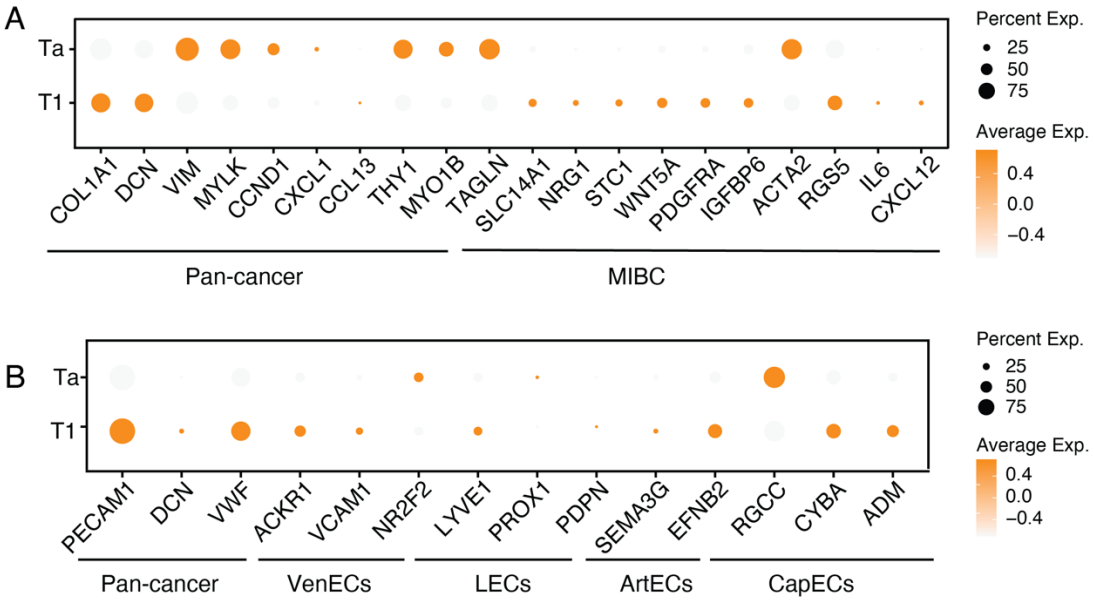

Fig. R4. Expression of markers in stromal cells

(A) Dot plot depicting the expression of pan-cancer and MIBC-specific fibroblast marker genes in fibroblasts from distinct stages. The size and color of the dots represent the percentage of cells expressing each gene and the average expression value, respectively. (B) Dot plot depicting the expression of marker genes for venous endothelial cells (VenECs), lymphatic endothelial cells (LECs), arterial endothelial cells (ArtECs), and capillary-like endothelial cells (CapECs) in endothelial cells from distinct stages.

8. Survival analysis/bulk cohorts -- the TCGA does not seem like the ideal cohort as the bladder TCGA tumors are from muscle-invasive bladder cancer. I would suggest to validate the clinical relevance of the gene programs in non-muscle invasive bladder cancer cohorts (e.g. UROMOL). Moreover, the extrapolation and validation of macrophage signatures in other TCGA cohorts e.g. kidney (Fig. 4) seems out of the scope of this manuscript, are not statistically very robust and distracts the flow of the paper.

Reply: We appreciate the reviewer's insightful comments and suggestions. Following the reviewer's recommendation, we investigated the UROMOL 2020 dataset, comprising 535 NMIBC patients with expression and clinical information. Regrettably, as the datasets are private, we have been unable to access them despite four requests. Consequently, we carried out validation using another large dataset containing 460 patients, which serves as the primary data source for the UROMOL NMIBC dataset[11]. Consistent with their prognostic impact in the TCGA MIBC datasets, both Macro_SPPI and IL4I1 are linked to poorer survival outcomes in the NMIBC dataset (Fig. R1). Additionally, to avoid distracting from the main findings of the paper, we have

eliminated the analyses of IL4I1 and SPP1 expression with clinical effects in TCGA cohorts. Furthermore, we moved the figure illustrating the association between IL4I1 expression and regulatory T-cell infiltration from Figure 4 to Supplementary Figure 4.

9. the tumor-immune interactions and results around NK/macrophage subpopulations are very interesting and quite novel in this disease context.

Reply: Thank you for emphasizing the tumor-immune interactions and results concerning NK/macrophage subpopulations. We appreciate your feedback.

10. Lastly, congratulate the authors for making the code available in GitHub. Please consider making the raw data publicly available to the international scientific community.

Reply: Thank you for your valuable suggestions. We have uploaded our raw count data to the Gene Expression Omnibus (GEO)[12]. Currently, the data is private and can be accessed via the website https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE277524 using the secure token 'stinmwygtrohjmr'. We promise to make the data publicly available immediately upon publication of the manuscript.

Due to policy restrictions, we are only able to upload the raw sequence data to the Genome Sequence Archive (GSA) repository, under accession number HRA005670, which can be accessed at https://ngdc.cncb.ac.cn/gsa-human/browse/HRA005670. We are currently in the process of applying to the Ministry of Science and Technology of China to make the data publicly accessible in the future, allowing users to access it without requiring a request.

Reference

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2. Ma, Z. *et al.* Interferon-dependent SLC14A1+ cancer-associated fibroblasts promote cancer stemness via WNT5A in bladder cancer. *Cancer Cell* **40**, 1550-1565.e7 (2022).
3. Chen, Z. *et al.* Single-cell RNA sequencing highlights the role of inflammatory cancer-associated fibroblasts in bladder urothelial carcinoma. *Nat Commun* **11**, 5077 (2020).
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6. Korsunsky, I. *et al.* Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat Methods* **16**, 1289–1296 (2019).
7. Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* **36**, 411–420 (2018).
8. Zheng, L. *et al.* Pan-cancer single-cell landscape of tumor-infiltrating T cells. *Science* **374**, abe6474 (2021).
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12. Wilhite, S. E. *et al.* NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Research* **41**, D991–D995 (2012).