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Peer Review File

Single-Cell and Spatial Transcriptomic Profiling Reveals Distinct Tumor Microenvironment Dynamics in Cervical Adenocarcinoma and Squamous Cell Carcinoma

Corresponding Author: Dr Huiling Shang

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Decision Letter:

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Dear Dr Shang,

Your manuscript entitled "Single-Cell and Spatial Transcriptomic Profiling Reveals Distinct Tumor Microenvironment Dynamics in Cervical Adenocarcinoma and Squamous Cell Carcinoma" has now been seen by 2 referees, whose comments are appended below. You will see from their comments copied below that while they find your work of potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be interested in considering a revised version that addresses these concerns.

We hope you will find the referees' comments useful as you decide how to proceed. Should further experimental data or analysis allow you to address these criticisms, we would be happy to look at a substantially revised manuscript. In particular, please note that the following revisions would be necessary for us to contact our referees again:

1 – Please address the concerns from reviewer #1 about the HPV infection.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if you wish to discuss the revision or if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If you decide to submit a revised version, we ask that your manuscript complies with our editorial policies. Specifically:

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It’s mandatory to provide access to the numerical source data for graphs and charts either through a repository or by providing the data in a Supplementary Data file (in excel format).

All blots/gels must be accompanied by size markers in every figure panel. Uncropped and unedited blot/gel images must be included as Supplementary Figure(s) in the Supplementary Information pdf.

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Please use the following link to submit your revised manuscript, point-by-point response to the referees' comments (which should be in a separate document to any cover letter) and any completed checklist:

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We hope to receive your revised manuscript within 3 months, but appreciate that every situation is unique. Please take as long as necessary to address these concerns in full, including performing any additional experimental work required. We look forward to receiving your revised manuscript when it is ready and will not enforce any specific deadline. However, please bear in mind that if the revision process takes significantly longer than six months, we may take into account whether anything similar has been accepted for publication at Communications Biology or published elsewhere in the meantime.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further. Thank you for the opportunity to review your work.

Best regards,

Johannes Stortz, PhD
Senior Editor
Communications Biology
orcid.org/0000-0002-5928-1850

on behalf of

Bibekanand Mallick
Editorial Board Member
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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I read with interest the manuscript titled "Single-Cell and Spatial Transcriptomic Profiling Reveals Distinct Tumor Microenvironment Dynamics in Cervical Adenocarcinoma and Squamous Cell Carcinoma", submitted by Dr. Shang and colleagues for publication in Communications Biology.
The authors use scRNA-sequencing and spatial transcriptomics to compare the tumor microenvironment in cervical adenocarcinoma and squamous cell carcinoma. These two histological subtypes have been recognized for more than a century and represent the classic pathological dichotomy of cervical cancer. It is well established that squamous cell carcinomas carry a somewhat worse prognosis overall than adenocarcinomas, although the reason for this remains unclear. The present study aims to explore key transcriptomic differences in these two subtypes. It is a purely descriptive report with no functional validation experiments. In addition, no external validation (e.g. by histological analysis with IF) was performed. The manuscript is well-written and structured. The topic is of clinical and basic scientific relevance. However, I have several concerns, both major and minor, that should be addressed by the authors.

Major concerns:

- The authors do not account for HPV infection. It has been well established that HPV-positive tumors are more immunogenic and associated with a better prognosis than non-HPV-related cancers. At the same time, there is a higher rate of non-HPV-related cancers among adenocarcinomas. I wonder, therefore, if the difference observed between the two subtypes could be related to HPV status? The authors should add this information to Supplementary Table 1 and address it in their results/discussion section.

- The authors included 2 patients who had adenosquamous carcinoma. This very small sample size precludes any meaningful conclusions. Furthermore, it is very confusing that this group is included in some analyses, but not in the majority. I would suggest removing all ASC data from the main figures and condensing this analysis into a single figure in the supplement.

- Can the authors elaborate on their thinking, how the high abundance of plasma cells in SCC might affect anti-tumor immunity? What is their role in the TME?

- Likewise – what do the authors think is the relevance of the close proximity between M2-macrophages and plasmablasts?

- Antibody-mediated immunity, which the authors invoke in line 400, is not a well-established concept in solid tumors. However, it has been shown that antibodies can effectively target viral antigens. Could this be a hint that different HPV types were at play in the SCC cases compared to the AC cases?

- Did the authors attempt to validate their Xenium cell segregation in the scRNA dataset? This would significantly increase the robustness of their findings.

- The authors invoke different responses to treatment
- Myeloid cell recruitment to the TME has been associated with immunosuppression, also in cervical cancer (Myeloid Derived Suppressor Cells). This should be discussed in the context of the authors' data.

- The authors suggest that the combination of an immune checkpoint inhibitor with an ICOS/IDO1 inhibitor could improve response rates. Such a statement warrants validation (e.g., by investigating the expression of ICOS/IDO1 in other cohorts such as the TCGA database and by confirming its association with inferior survival). A more explicit discussion on how these findings might stratify patients for immunotherapy would strengthen the translational impact.

- The restricted gene panel (430 genes) in the Xenium platform is acknowledged but could be more explicitly discussed in terms of its impact on detecting rare cell populations.

- The permutation-based spatial distance analysis is well-described, but the rationale for selecting certain cell types as "index cells" could be more explicit.

Minor concerns:
- In Figure 2d, I assume the arrowheads on the x-axis code for the carcinoma subtype? Please provide a legend! I assume that "green" refers to "adenosquamous carcinoma"? In Fig. 2 b and 2h, green codes for adenocarcinoma. Please ensure consistency! Same with Fig. 2g.

- A few minor spelling corrections should be done:
o Line 358: "It is" instead of "it's".
o Line 465: "that" instead of "the"
o Line 470: "risk" instead of "rick"

- Line 389: What do the authors mean by "unique clinical behavior"?
- In the Xenium data, some subclusters were labeled as "other" due to insufficient markers. It may help to mention how future iterations could improve these annotations (e.g., expanded gene panels).

Reviewer #2 (Remarks to the Author):

Shang et al. performed a comprehensive single-cell and spatial transcriptomics analysis of cervical cancer, which reveals distinct tumor microenvironment landscapes between adenocarcinoma (AC) and squamous cell carcinoma (SCC) subtypes, including cellular composition, cell-cell interactions, and spatial structures. Below are some concerns:

The authors mentioned the inconsistency of major cell proportions in Xenium data. However, I notice that most samples have paired single-cell and spatial data. Therefore, the authors should comprehensively compare whether additional consistent findings exist between these two omics data to further demonstrate their alignment. For example, ICOS+ Tregs and their associated interactions were distinctively observed in the SCC microenvironment in scRNA-seq data. Can these features also be identified in the Xenium data?

The authors also need to validate their findings in other independent patient cohorts, such as performing cell deconvolution analysis using TCGA datasets to compare cellular differences between AC and SCC subtypes.

Throughout the manuscript, inconsistent terminology is used for single-cell RNA sequencing data ("scRNA-seq data" and "sc-RNA data"). Standardization to "scRNA-seq data" is required.

In Figure 2, label formatting is inconsistent: labels a-h use lowercase letters, while i-j use uppercase letters. Uniform formatting should be applied.

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Version 1:

Decision Letter:

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Dear Dr Shang,

Your manuscript entitled "Single-Cell and Spatial Transcriptomic Profiling Reveals Distinct Tumor Microenvironment Dynamics in Cervical Adenocarcinoma and Squamous Cell Carcinoma" has now been seen again by our referees, whose comments appear below. In light of their advice I am delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Biology.

We therefore invite you edit your manuscript to comply with our format requirements and to maximise the accessibility and therefore the impact of your work.

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It is important that you pay careful attention to the requests in these documents to avoid a delay in formal acceptance of the article.

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Congratulations on an excellent paper!

Best regards,

Johannes Stortz, PhD
Senior Editor
Communications Biology
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on behalf of

Bibekanand Mallick
Editorial Board Member
Communications Biology
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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have made significant and important changes to their manuscript and have addressed all my concerns adequatly. Even though I still think that the UMAPS in Fig. 1 would be better if they had excluded the adenosquamous carcinoma cases, this is a minor point and the manuscript is now OK to be published from my side. Great effort!!

Reviewer #2 (Remarks to the Author):

I have carefully reviewed the revised manuscript and the authors' response to my previous comments. The authors have adequately addressed all my previous concerns. I have no further comments and recommend acceptance of this manuscript.

General response to reviewers and editorial comments

In general, the referees have positive response to our manuscript, stating that the manuscript "is well-written and structured. The topic is of clinical and basic scientific relevance" (#1) and that our work “performed a comprehensive single-cell and spatial transcriptomics analysis of cervical cancer, which reveals distinct tumor microenvironment landscapes between adenocarcinoma (AC) and squamous cell carcinoma (SCC) subtypes” (#2).

In addition, the reviewers raise some important points regarding the limitations of the assay as presented, and the computational analyses of the data, which we have addressed by additional analysis. The main analyses include:

1) Sub-analyses stratifying HPV+ and HPV- cervical cancer,
2) Validation of Xenium cell segregation using the scRNA dataset,
3) More comprehensive analysis of the cell-cell interactions in Xenium data to validate the results found in scRNA-seq data,
4) Validation of immune cell proportion disparities using a public TCGA dataset.

In the following pages is our point-by-point response to all specific criticisms of the reviewers. The reviewers' comments are in *italic*, and our response to them is in Times Regular. Changes to the manuscript are indicated in **bold**.

Reviewer #1 (Remarks to the Author):

I read with interest the manuscript titled "Single-Cell and Spatial Transcriptomic Profiling Reveals Distinct Tumor Microenvironment Dynamics in Cervical Adenocarcinoma and Squamous Cell Carcinoma", submitted by Dr. Shang and colleagues for publication in Communications Biology.
The authors use scRNA-sequencing and spatial transcriptomics to compare the tumor microenvironment in cervical adenocarcinoma and squamous cell carcinoma. These two histological subtypes have been recognized for more than a century and represent the classic pathological dichotomy of cervical cancer. It is well established that squamous cell carcinomas carry a somewhat worse prognosis overall than adenocarcinomas, although the reason for this remains unclear. The present study aims to explore key transcriptomic differences in these two subtypes. It is a purely descriptive report with no functional validation experiments. In addition, no external validation (e.g. by histological analysis with IF) was performed.
The manuscript is well-written and structured. The topic is of clinical and basic scientific relevance. However, I have several concerns, both major and minor, that should be addressed by the authors.

Major concerns:

- The authors do not account for HPV infection. It has been well established that HPV-positive tumors are more immunogenic and associated with a better prognosis than non-HPV-related cancers. At the same time, there is a higher rate of non-HPV-related cancers among adenocarcinomas. I wonder, therefore, if the difference observed between the two subtypes could be related to HPV status? The authors should add this information to Supplementary Table 1 and address it in their results/discussion section.

We agree that HPV status is a critical factor. **We have now: (i) Added HPV status for all samples to Supplementary Data 1, (ii) Performed sub-analyses stratifying HPV+ and HPV− adenocarcinoma since all SCC samples are HPV+ (new Supplementary Figure 4a), (iii) Further compared the HPV+ AC and HPV+ SCC samples to find out the contribution of HPV infections to immunogenic tumor microenvironment (new Supplementary Figure 4b), (iv) Added a dedicated paragraph to the Results (p. 6, lines 244-257)**. The new analysis showed that the CD8 T cells and NK cells were more enriched in HPV+ AC, while Tregs were more enriched in HPV- AC. However, the proportions of plasma cells were not statistically different, slightly higher in HPV- AC, implying that the immunogenic tumor microenvironment was not correlated with HPV infection, but more related to the SCC and AC cancer types. When comparing HPV+ AC and HPV+ SCC samples regardless of HPV status, we also found more immunogenic microenvironment in HPV+ SCC samples.

- The authors included 2 patients who had adenosquamous carcinoma. This very small sample size precludes any meaningful conclusions. Furthermore, it is very confusing that this group is included in some analyses, but not in the majority. I would suggest removing all ASC data from the main figures and condensing this analysis into a single figure in the supplement.

Per the reviewer's suggestion: **We removed all downstream analysis including ASC samples from the main figures (Figure 1d, f, h and j)** but still keep the UMAPs (Figure 1c and g), as we want to introduce a more comprehensive atlas about cervical cancer; **(ii) ASC analyses are consolidated into Supplementary Figure 4c and d with a brief description in the Results (p. 7, lines 258-266).** The main text now explicitly focuses on AC vs. SCC comparisons.

- Can the authors elaborate on their thinking, how the high abundance of plasma cells in SCC might affect anti-tumor immunity? What is their role in the TME?

We thank the reviewers for the insightful comments. **We have expanded the Discussion in details (p. 14, lines 613-623)**: In cervical SCC, high plasma cell abundance may have dual functions: the IgG secreted plasma cells may promote antibody-dependent anti-tumor responses via viral antigen targeting (e.g., HPV E6/E7), these antibodies may neutralize the viral particles to inhibit infection of new cells, or opsonize tumor cells through phagocytosis (ADCP) or NK-cell killing (ADCC). Conversely, diffuse intratumoral plasma cells (IgA/IgG4-dominated) drive immunosuppression through M2 macrophage polarization, fibrosis, and T-cell exclusion. Spatial distribution, antibody isotype, and HPV antigen specificity determine net impact.

- Likewise – what do the authors think is the relevance of the close proximity between M2-macrophages and plasmablasts?

The spatial proximity between M2-macrophages and IgG2+ plasmablasts in SCC may be due to the mechanism that CD163+ M2 macrophages induce differentiation and isotype switching of plasma cells (reference, https://pmc.ncbi.nlm.nih.gov/articles/PMC3457728/). **We included the content in the Results (p. 10, line 425) and cited related reference.**

- Antibody-mediated immunity, which the authors invoke in line 400, is not a well-established concept in solid tumors. However, it has been shown that antibodies can effectively target viral antigens. Could this be a hint that different HPV types were at play in the SCC cases compared to the AC cases?

We agreed with what the reviewer stated about the concept. **We removed the statement with "Antibody-mediated immunity" to avoid misunderstanding on p. 12, line 481.** After subgrouping the HPV+ and HPV- samples in cervical AC, we reanalyzed the data and didn't find plasma cells or IgG+ plasma cells were more abundant in HPV+ AC samples than in HPV- AC samples (**Supplementary Figure 4a**), but the difference was observed when comparing HPV+ AC and HPV+ SCC samples (**Supplementary Figure 4b**). Based on these results, we would assume the high abundance of plasma cells in SCC was not mainly caused by HPV infections, but more associated with the cancer types.

- Did the authors attempt to validate their Xenium cell segregation in the scRNA dataset? This would significantly increase the robustness of their findings.

Yes we performed the validation analysis by integrating both datasets and compared cell type annotations based on different modalities (p. 4, lines 145-151 and p. 5, lines 191-196, Supplementary Figure 2 and Supplementary Figure 3c-e): the Xenium cell-type assignments were cross-validated against scRNA-seq using label transfer. High concordance was observed (≥50% accuracy) for many major cell types including epithelial cells, endothelial cells, fibroblasts, CAFs and NK/T lymphocytes.

- The authors invoke different responses to treatment
- Myeloid cell recruitment to the TME has been associated with immunosuppression, also in cervical cancer (Myeloid Derived Suppressor Cells). This should be discussed in the context of the authors' data.

Per the reviewer's suggestion: **We included analysis about the MDSCs using our data. Our analysis revealed significantly higher expression of MDSC marker genes S100A8/9 in AC samples, suggesting their potential role in driving poor patient prognosis (p. 6, lines 239-243, Supplementary Fig. 3g).**

- The authors suggest that the combination of an immune checkpoint inhibitor with an ICOS/IDO1 inhibitor could improve response rates. Such a statement warrants validation (e.g., by investigating the expression of ICOS/IDO1 in other cohorts such as the TCGA database and by confirming its association with inferior survival). A more explicit discussion on how these findings might stratify patients for immunotherapy would strengthen the translational impact.

We thank the reviewer for the comments. Our statement was based on the spatial proximity of ICOS+ Tregs, IDO1+ CAFs and activated CD8+ T cells within the microenvironment. The expression levels however represent the bulk information without spatial information. Therefore it's hard to validate the statement using TCGA

dataset. **We rephrased the sentences and discuss more on how our results could stratify patients for immunotherapy in future, especially the identification of spatial neighborhoods with different functionalities (p. 16, lines 669-675).**

- The restricted gene panel (430 genes) in the Xenium platform is acknowledged but could be more explicitly discussed in terms of its impact on detecting rare cell populations.

We added the limitations in the Discussion (p. 16, lines 680-687): The 430-gene panel may underdetect rare populations (e.g., γδ T cells). The customized panels and other single-cell level data (e.g., spatial proteomic data, scRNA-seq data) are needed to identify more cell types/subtypes which are biologically meaningful.

- The permutation-based spatial distance analysis is well-described, but the rationale for selecting certain cell types as "index cells" could be more explicit.

We clarified this in Results (p. 10, line 408) and Methods sections (p. 19, lines 824-837): In our analysis, all ~50 annotated cell types were selected as "index cell" each time, using permutation distance analysis, we identified its most proximal cell types as potential interaction partners.

Minor concerns:
- In Figure 2d, I assume the arrowheads on the x-axis code for the carcinoma subtype? Please provide a legend! I assume that "green" refers to "adenosquamous carcinoma"? In Fig. 2 b and 2h, green codes for adenocarcinoma. Please ensure consistency! Same with Fig. 2g.

We thank the reviewer for the comments. The arrowheads on the x-axis represent the cancer subtypes. **We changed the colors in Figure 2d, 2g as suggested, and changed the Figure 1f, 1j for consistency, the figure legends were modified accordingly (p. 27, line 1132; p. 26, line 1085).**

- A few minor spelling corrections should be done:
o Line 358: "It is" instead of "it's".
o Line 465: "that" instead of "the"
o Line 470: "risk" instead of "rick"

We corrected the spelling errors throughout the manuscript.

- Line 389: What do the authors mean by "unique clinical behavior"?

We were mainly referring to: high link to the HPV infections driving carcinogenesis and therapeutic responsiveness that may be associated with the distinct immune

microenvironment of SCC. **We rephrased the sentence after removing "unique clinical behavior" for appropriate wording (p. 11, line 467).**

- In the Xenium data, some subclusters were labeled as "other" due to insufficient markers. It may help to mention how future iterations could improve these annotations (e.g., expanded gene panels).

We thank the reviewer for the comments. **We added more discussions about the annotations (p. 16, line 682).** We will take advantage of customized panels and other single-cell level data (e.g., spatial proteomic data, scRNA-seq data) to incorparate with the ST data to detect more cell types/subtypes.

Reviewer #2 (Remarks to the Author):

Shang et al. performed a comprehensive single-cell and spatial transcriptomics analysis of cervical cancer, which reveals distinct tumor microenvironment landscapes between adenocarcinoma (AC) and squamous cell carcinoma (SCC) subtypes, including cellular composition, cell-cell interactions, and spatial structures. Below are some concerns:

The authors mentioned the inconsistency of major cell proportions in Xenium data. However, I notice that most samples have paired single-cell and spatial data. Therefore, the authors should comprehensively compare whether additional consistent findings exist between these two omics data to further demonstrate their alignment. For example, ICOS+ Tregs and their associated interactions were distinctively observed in the SCC microenvironment in scRNA-seq data. Can these features also be identified in the Xenium data?

We thank the reviewer for the precious comments. Per the reviewer's suggestion, **we performed comprehensive analysis to compare the findings about cell-cell interactions of the six major cell identities showed in Figure 4b-f (including Tregs and pDCs) from both omics datasets.** First we cross-checked the consistency of cell annotations, especially CCL4 CTLs (ST data) were most similar with activated CD8 T/NK (scRNA-seq data) based on our integrative analysis (Supplementary Figure 7a). Our distance analysis revealed stronger interactions among these cell types in SCC samples, especially pDC- Treg and aCD8 T/NK-Treg pairs (Supplementary Figure 7b). The results are consistent with the Cellchat results based on scRNA-seq data, **we added one paragraph about the result on p. 12, lines 495-504.**

The authors also need to validate their findings in other independent patient cohorts, such as performing cell deconvolution analysis using TCGA datasets to compare cellular differences between AC and SCC subtypes.

As the reviewer suggested, we performed deconvolution (CIBERSORTx) on TCGA-CESC data to validate our results: SCC showed significantly higher plasma cells, CD8 T cells and CD8 T/NK cells infiltration than AC samples. **We have now included the results in related Results section on p. 6, lines 223 (Supplementary Figure 3f).**

Throughout the manuscript, inconsistent terminology is used for single-cell RNA sequencing data ("scRNA-seq data" and "sc-RNA data"). Standardization to "scRNA-seq data" is required.

We have uniformly changed to "scRNA-seq data" throughout.

In Figure 2, label formatting is inconsistent: labels a-h use lowercase letters, while i-j use uppercase letters. Uniform formatting should be applied.

All panel labels were standardized to lowercase (a–j).

We thank the reviewers again for their insightful comments, and feel that the extensive revisions to the manuscript have addressed the technical issues raised. We also hope that the changes have made the work and the advance it represents more clear and precise to a general audience.

Sincerely, Shang et al.