

Dear Editors and Reviewers:

Thanks for your letter and the reviewer comments concerning our manuscript entitled "CLTC Expression in Oral Squamous Cell Carcinoma: Insights into Prognostic Value and Immune Microenvironment Modulation" (Manuscript ID: 46057fb5-5927-4307-9864-1e7ae5b308ba). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction, which we hope meet with approval. In addition, regarding to the reviewers' insightful comments, the answers were listed one-by-one as follows and the correspondingly revised information marked in red could be found at the revised manuscript. Thanks again.

Editor comments:

Dear Dr Yang,

Your manuscript, "CLTC Expression in Oral Squamous Cell Carcinoma: Insights into Prognostic Value and Immune Microenvironment Modulation", has now been assessed.

We invite you to revise your paper, carefully addressing the comments from the reviewers and the editor. Please ensure the results are accurately reported, any overstated conclusions are rewritten and the limitations of the work fully explained. When your revision is ready, please submit the updated manuscript and a point-by-point response. This will help us move to a swift decision.

We recommend submitting all revisions within the mentioned deadline.

If you need more time, please contact us and include your submission ID.

Kind regards,

Manoj Pandey

Editor

World Journal of Surgical Oncology

Support contact: Reginald.Medina@springernature.com

Submission ID: 46057fb5-5927-4307-9864-1e7ae5b308ba

Response: Thank you very much for your message and for the opportunity to revise our manuscript. We sincerely appreciate the time and effort of the editor and reviewers in evaluating our work and providing constructive comments. We will carefully revise the manuscript according to the reviewers' and editor's suggestions, ensure that the results are accurately reported, revise any overstated conclusions, and further clarify the limitations of the study. We also prepared a detailed point-by-point response to all comments. Thank you again for your consideration.

Reviewer comments:

Reviewer #1:

1. CLTC is associated with poor prognosis and ECM activation, yet GSEA shows negative enrichment in key oncogenic pathways (MAPK, cancer pathways). Please reconcile this contradiction or re-evaluate the enrichment analysis.

Response: We thank the reviewer for this comment. We would like to clarify that our manuscript did not conclude that CLTC activates the ECM pathway. The DEG-based KEGG enrichment analysis only indicated that genes differentially expressed between the CLTC-high and CLTC-low groups were enriched in ECM-receptor interaction and related pathways. This analysis identifies pathway involvement among DEGs, but does not determine pathway activation or inhibition.

We re-checked the GSEA input and ranking direction. GSEA was performed using the complete ranked gene list from the CLTC-high versus CLTC-low comparison, without applying a differential-expression cutoff. Since the CLTC-high group was used as the experimental group and the CLTC-low group as the reference group, a negative NES indicates that the corresponding pathway is preferentially enriched in the CLTC-low phenotype.

Therefore, the negative enrichment of ECM-receptor interaction, MAPK signaling, and pathways in cancer does not contradict the KEGG results. The KEGG analysis suggests that these pathways are involved in the transcriptional differences between the two CLTC expression groups, whereas GSEA provides directional information showing that they are not positively enriched in the CLTC-high group.

We have revised the manuscript to make this interpretation clearer and to avoid any implication that CLTC directly activates ECM-related or other oncogenic pathways.

2. You report reduced CD8⁺ T cells (immunosuppression) alongside increased TME/immune checkpoint scores (immunoactivation). Provide a unified interpretation or stratified analysis to resolve this inconsistency.

Response: Thanks for your constructive comments. We sincerely thank the reviewer for pointing out this apparent inconsistency. After carefully rechecking the original data, group annotations, and figure preparation process, we found that the colors representing the CLTC-high and CLTC-low groups in Figure 6E-H had been inadvertently reversed during figure preparation. Specifically, the CLTC-high group should be shown in pink and the CLTC-low group in blue, consistent with the group annotation used in the ssGSEA analysis.

This color-labeling error also led to an incorrect description of the corresponding immune-related results in the original manuscript. After correcting the figure and re-examining the analysis results, we confirmed that CD8⁺ T-cell infiltration, TME scores, and immune checkpoint-related scores showed consistent trends between the two CLTC expression groups. Specifically, compared with the CLTC-low group, the CLTC-high group exhibited lower CD8⁺ T-cell infiltration as well as reduced TME and immune checkpoint scores.

We have redrawn Figure 6E-H with the correct color annotations and revised the corresponding description in Section 3.3 of the manuscript. All related modifications have been highlighted in red.

3. The statement that 5-FU and paclitaxel "exert anticancer effects through binding to CLTC" is not supported by docking data alone. Remove or significantly tone down this claim.

Response: Thanks, we have substantially toned down this statement in the Discussion section. As highlighted in red in the revised Discussion, the docking results suggest potential structural interactions; however, we explicitly state that further functional

experiments are required to verify whether this binding plays any role in the pharmacological effects of the drug or in mechanisms of drug resistance.

Reviewer #2:

This study comprehensively investigates CLTC as a prognostic biomarker and immune regulator in oral squamous cell carcinoma (OSCC) using multi-omics, bioinformatics, and in vitro functional experiments. The topic is clinically important, the design is systematic, and the core findings are consistent. However, major revisions are required to resolve inconsistencies, improve rigor, standardize figures, clarify methodology, and polish presentation.

1. Fix critical naming inconsistency (severe error)

Unify CLTC throughout the manuscript. Repeatedly miswritten as CTLC in Figures 3, 4, captions, and text. Must be corrected in all panels, tables, and legends.

Response: Thanks, we sincerely thank the reviewer for pointing out this critical naming inconsistency. We apologize for the error. We have carefully checked the entire manuscript and corrected all instances of the incorrect term CTLC to the correct gene symbol CLTC. The corrections have been made throughout the main text, Figures 3 and 4, figure captions, tables, and legends. We have also rechecked all panels and annotations to ensure that CLTC is used consistently throughout the revised manuscript.

2. Upgrade all figures to publication quality

All figures are blurry, misaligned, missing labels, or unreadable.

Redraw all plots with clear axes, titles, P values, n, units, and scale bars

Add full, uncropped Western blot images with molecular weights (Figure 11)

Remove garbled text and distorted heatmaps; standardize colors and fonts

Label all spatial plots, t-SNE/UMAP, and cell communication diagrams

Response: Thanks, we have redrawn or reformatted the figures and improved their resolution. High-resolution PDF versions of the figures have also been uploaded to the submission system. In addition, we added and clearly labeled the axes, titles,

group labels, legends, statistical annotations, P values, sample sizes, units, and scale bars where applicable. We also corrected layout misalignment issues, removed garbled or distorted text, and standardized the fonts across all figures. For the heatmaps, enrichment analysis plots, survival curves, immune infiltration plots, drug sensitivity plots, t-SNE/UMAP plots, and cell-cell communication plots, we revised the annotations to ensure that all cell types, groups, pathway names, and interaction axes are clearly readable. Regarding the Western blot data, the supplementary file now includes complete, uncropped Western blot images of the proteins, labeled with molecular weights.

3. Resolve contradictory immune microenvironment conclusions

Text and figures conflict on the immune cell direction:

Correct: CLTC high correlates with low CD8⁺ T cells, low cytotoxicity

Remove contradictory claims about "macrophages/neutrophils."

Clarify that CLTC promotes immune exclusion and suppression

Response: Thanks, we have revised the relevant content throughout the manuscript to clearly state that high CLTC expression is associated with reduced CD8⁺ T-cell infiltration and decreased cytotoxic activity, suggesting that it may impair antitumor immune surveillance. Meanwhile, we have removed the contradictory statements regarding macrophages/neutrophils to avoid inconsistent interpretations of the immune microenvironment. In addition, we have further clarified in the Abstract, Section 3.3 of the Results, the Discussion, and the Conclusion, with changes highlighted in red, that high CLTC expression does not indicate immune activation, but is instead associated with immune exclusion and remodeling of an immunosuppressive tumor microenvironment. In the revised manuscript, the conclusions regarding the immune microenvironment have been unified as follows: high CLTC expression promotes immune exclusion and immunosuppression in OSCC, which may partially explain its association with poor prognosis.

4. Strengthen experimental rigor and missing data

Add the following essential experiments/information:

Quantification of Western blot bands (Figure 11A–C)

Transwell migration without Matrigel to separate migration vs invasion

Rescue assay to confirm CLTC specificity

IHC validation of CLTC in OSCC tissues

Repeat functional assays in a second cell line (e.g., HSC3 or CAL27)

Response: We sincerely thank the reviewer for these important suggestions, which are very helpful for improving the rigor and reliability of our experimental validation. In response to this comment, we have made the following revisions and additions.

First, we have added quantitative analysis of the Western blot bands in Figure 10A-C. The relative CLTC protein levels were quantified using ImageJ and normalized to β -actin. The corresponding statistical results have now been included in the revised Figure 10, which further supports the upregulation of CLTC in OSCC tissues and cell lines, as well as the knockdown efficiency of si-CLTC#1 and si-CLTC#2.

Second, to strengthen the reproducibility of the functional results, we repeated the CLTC knockdown experiments in a second OSCC cell line, HSC3. Consistent with the results observed in SCC25 cells, CLTC knockdown in HSC3 cells suppressed cell proliferation, colony formation, migration, and invasion. These additional data have been added as supplementary results and are presented in Figure S1.

Regarding the Transwell migration assay without Matrigel, we agree that this assay can provide additional evidence to distinguish cell migration from invasion. In the current study, we used the wound healing assay to evaluate migratory ability and the Matrigel-coated Transwell assay to assess invasive capacity. Thus, migration and invasion were evaluated by two commonly used but distinct functional assays. Considering the current scope of the revision and the fact that we have already added validation in a second OSCC cell line, we did not further perform an additional non-Matrigel Transwell migration assay.

Regarding the rescue assay, we agree that rescue experiments would provide stronger evidence for the specificity of CLTC knockdown. In this study, to reduce the

possibility of siRNA off-target effects, we used multiple siRNAs targeting CLTC and selected two effective siRNAs for functional validation. The consistent inhibitory effects observed after CLTC knockdown, together with the newly added validation in HSC3 cells, support the biological relevance of CLTC in OSCC malignant phenotypes. However, we acknowledge that rescue experiments would further strengthen the causal evidence, and we have added this point to the limitations of the study.

Regarding IHC validation, we agree that immunohistochemical staining in OSCC tissues would provide valuable spatial and pathological evidence for CLTC protein expression. In the present study, we validated CLTC protein upregulation in paired OSCC and adjacent normal tissues by Western blot. Because additional tissue collection, staining optimization, and standardized pathological scoring could not be completed within the current revision period, we did not perform IHC validation at this stage. We have acknowledged this limitation in the revised Discussion and stated that future studies should include IHC analysis in larger clinical cohorts to further validate the clinical and pathological significance of CLTC expression in OSCC.

Accordingly, we have revised the Results, Figure 10, supplementary Figure S2, and the Discussion section to more accurately present the strengthened experimental validation and the remaining limitations.

5. Standardize statistical reporting

Report exact P values, 95% CIs, and FDR for all analyses

Add number-at-risk tables to all Kaplan–Meier curves

Justify $|\log FC| > 0.5$ for DEGs; explain low stringency

Specify cutoff for high/low CLTC expression

Response: Thanks for your constructive comments. The p values, 95% confidence intervals (CIs), and FDR values involved in the statistical analyses have been provided in Supplementary Material. Risk tables were added to the Kaplan-Meier survival curves. Patients with oral cancer were divided into high- and low-expression groups based on the median CLTC expression level, which has been clarified in the

Methods section. In addition, the information on differentially expressed genes identified between the CLTC high- and low-expression groups has also been presented in Supplementary Material.

6. Improve nomogram and prognostic modeling

Remove redundant variables (Stage already includes T/N/M)

Report full Cox coefficients, C-index, and bootstrap validation

Compare nomogram performance with AJCC staging alone

Fix overlapping labels and unreadable scales (Figure 4C, 4F)

Response: Thanks for your constructive comments. Thanks for your constructive comments. Thank you for your constructive comments. We performed univariate Cox regression analysis, which showed that CLTC, age, stage, T stage, and N stage were prognostic factors for oral cancer; therefore, stage was retained in the model (Figure 4B). To compare the nomogram with AJCC staging alone, we constructed a nomogram model based on stage, T stage, and N stage. The results showed that the nomogram had higher predictive accuracy than the AJCC staging system alone, as indicated by AUC and C-index values. These results are presented in Figure S1. The Cox regression coefficients have been added to Table S1-S2. In addition, we redrew the nomogram to address the overlapping labels and unreadable scales in the original figure (Figure 4C, Figure 4F).

7. Clarify scRNA-seq & spatial transcriptomics

Correct cell annotation: remove chondrocytes/tissue stem cells (not relevant for OSCC)

Show CLTC expression specifically in malignant epithelial cells

Explain why monocyte-related modules are central to CLTC function

Remove irrelevant or uninterpretable hdWGCNA plots (Figure 8)

Response: Thanks for your constructive comments. First, we re-examined the cell annotation strategy for the scRNA-seq analysis and removed inappropriate cell-type annotations, including chondrocytes and tissue stem cells, because these cell types are not relevant to OSCC. The revised cell annotation now focuses on OSCC-associated

cell populations, including epithelial cells, inflammatory epithelial cells, CAFs, myCAFs, endothelial cells, pericytes, T cells, plasma cells, dendritic cells, macrophages, and inflammatory macrophages. The updated cell annotations were validated using canonical marker genes and are presented in the revised Figure 7A-B.

Second, we further clarified the expression distribution of CLTC at the single-cell level. In the revised analysis, we showed the expression pattern of CLTC across OSCC-associated cell populations. The results indicated that CLTC was relatively highly expressed in epithelial cells and inflammatory epithelial cells, and also showed elevated expression in macrophages, inflammatory macrophages, and endothelial cells. Because epithelial cells and inflammatory epithelial cells represent the tumor epithelial compartment within OSCC tissues, these findings support the enriched expression of CLTC in malignant epithelial-associated cell populations. We have described this in the revised Results section and in the legend of Figure 7D.

Third, we refined and corrected the interpretation of the macrophage/monocyte-related findings. We no longer overemphasize the monocyte-related module identified by hdWGCNA, but instead shift the focus to ligand-receptor based cell-cell communication analysis. The revised results showed extensive interactions between CLTC-high macrophages and epithelial cells, CAFs, endothelial cells, T cells, inflammatory epithelial cells, and inflammatory macrophages. Key communication axes included MIF-CD74/CD44, MIF-CD74/CXCR4, and SPP1-related interactions, suggesting that macrophage-associated signaling may participate in inflammatory regulation, chemotactic recruitment, cell adhesion, and extracellular matrix remodeling within the OSCC microenvironment. These findings provide a clearer biological basis for the relationship between macrophage/monocyte-related programs and CLTC-associated remodeling of the immune microenvironment.

Finally, in response to the reviewer's suggestion, we removed the original hdWGCNA figures that were unrelated or difficult to interpret. The original Figure 8 has been replaced with a more interpretable macrophage-centered cell-cell communication analysis, including heatmaps of the number and strength of

intercellular interactions, bubble plots of ligand-receptor interactions when macrophages act as sender or receiver cells, and violin plots showing the expression of genes related to the MIF, CXCL, and SPP1 signaling pathways. The corresponding figure legends and Results section have also been revised accordingly.

8. Fix molecular docking and drug sensitivity

Clarify: high CLTC = resistant to paclitaxel/cisplatin

Report proper binding energy thresholds (< -7.0 kcal/mol = strong binding)

Do NOT overclaim CLTC as a “direct drug target” without biochemical validation

Response: Thanks for your constructive comments. We sincerely thank the reviewer for these valuable comments. We have revised the relevant content in Section 3.5 accordingly. Specifically, we clarified that high CLTC expression was associated with predicted resistance to paclitaxel and cisplatin. We also revised the interpretation of the molecular docking results and stated that binding energies lower than -7.0 kcal/mol indicate relatively strong binding potential. In addition, we removed overstatements suggesting that CLTC is a direct drug target and emphasized that these computational findings require further biochemical and experimental validation. All corresponding revisions have been made in Section 3.5 and highlighted in red.

9. Expand transparent limitations

Add:

Retrospective data; single-center design

No in vivo animal experiments

No external clinical validation cohort

No mechanistic data on how CLTC regulates MAPK/PI3K

Correlative only; causality unproven

Response: Thank you for this valuable suggestion. We agree that the limitations of the present study should be stated more transparently to avoid overinterpretation of the findings. The current study was designed mainly as a multi-omics bioinformatics analysis combined with preliminary experimental validation, rather than a full mechanistic or preclinical study. Therefore, in vivo animal experiments, external

clinical cohort validation, and detailed mechanistic studies on how CLTC regulates MAPK/PI3K-related signaling were beyond the scope of the current work. In the revised manuscript, we have expanded the “Limitations” section to explicitly acknowledge that:

1. the clinical data were mainly retrospective and partly derived from public databases;
2. the validation was limited by a single-center tissue cohort;
3. no in vivo animal experiments were performed;
4. no independent external clinical validation cohort was included;
5. the mechanistic relationship between CLTC and MAPK/PI3K signaling remains to be experimentally clarified; and
6. some findings are correlative, and causality cannot be fully established based on the current data.

We believe this revision makes the interpretation of our findings more cautious and transparent. Future studies with multicenter clinical cohorts, animal models, and pathway-specific mechanistic experiments will be needed to further confirm the causal role and regulatory mechanism of CLTC in OSCC progression.

10. Polish academic English and formatting

Italic CLTC uniformly

Correct typos, run-on sentences, and inconsistent abbreviations

Remove repetition in Results and Discussion

Shorten overly long paragraphs and improve logical flow

Response: Thanks for your constructive comments. We have systematically polished the manuscript for academic English and standardized the formatting throughout. The revisions focused on correcting grammatical errors, spelling mistakes, inconsistencies in capitalization, run-on sentences, and nonstandard abbreviations. We also harmonized the terminology and replaced potentially misleading expressions such as “high/low risk groups” with “expression-based grouping”. In addition, we streamlined repetitive descriptions of statistical results and figure contents in the Results section.

We also shortened overly lengthy literature-review-style paragraphs in the Discussion, making this section more focused on the interpretation of our findings, the significance of the study, and its limitations.

11. Some similar Oral Squamous Cell Carcinoma studies should be referenced. For example, PMID: 41884849, 41497890, 41142627, 41122645.

Response: Thanks for your constructive comments. We have added recent references related to PPME1, BEND3, CLIC6, and CORO1A in the first paragraph of the Introduction, with the newly added text highlighted in red. These studies performed pan-cancer analyses using public databases such as TCGA and GEO, combined with prognostic evaluation, immune microenvironment analysis, and experimental validation. Collectively, these studies demonstrate the importance of integrating multi-omics analyses with functional validation to identify potential diagnostic, prognostic, and immune-related biomarkers. They also provide important methodological references for the design of the present study.

12. Recent literature should be cited, and the format of the references needs to be further optimized.

Response: Thanks for your constructive comments. We have added recent references related to PPME1, BEND3, CLIC6, and CORO1A in the Introduction section. These studies adopted integrative analytical strategies similar to those used in the present study, including analyses of public databases such as TCGA and GEO, pan-cancer expression profiling, prognostic evaluation, immune microenvironment correlation analysis, functional enrichment analysis, and experimental validation. These findings indicate that multi-omics analysis combined with experimental validation is an important approach for identifying diagnostic, prognostic, and immune-related biomarkers in cancer. In the revised manuscript, we further state that these studies provide methodological references and theoretical support for the present study, in which we systematically investigated the role of CLTC in OSCC from multiple perspectives, including expression characteristics, prognostic value, immune microenvironment regulation, and functional validation.

13. The authors should consider engaging in a professional language editing service to ensure the clarity and coherence of the manuscript.

It was a pleasure reading your paper, "CLTC Expression in Oral Squamous Cell Carcinoma: Insights into Prognostic Value and Immune Microenvironment Modulation," and I thank you for your work. Please address the issues mentioned, as they can enhance the contribution of manuscripts to the field. The findings will fill a key gap in our understanding of cancer prognosis and provide new tools for cancer treatment to improve patient outcomes.

Response: We have systematically polished the English language and carefully proofread the grammar throughout the manuscript. We focused particularly on revising paragraphs with unclear expressions, overly lengthy sentence structures, or insufficiently smooth logical transitions. In addition, we further standardized the use of key terms throughout the manuscript, including CLTC, OSCC, immune microenvironment, scRNA-seq, and spatial transcriptomics, to ensure accuracy and consistency. We sincerely thank the reviewer again for the positive evaluation and valuable comments. These revisions have helped further improve the quality and academic contribution of our manuscript.

Reviewer #3:

The manuscript investigates CLTC expression in oral squamous cell carcinoma using multi omics data and experimental validation. This is a rigorous and well-structured manuscript, but I have one question: why did the author choose the CLTC gene for this study? While the vast majority of the manuscript content meets the publication criteria of this journal, several details issues need to be addressed before publication can be recommended. My concerns are outlined below.

(1) In Section 3.3, the authors report that CLTC expression is negatively correlated with CD8⁺ T cell infiltration, which implies an immunosuppressive role. However, within the same subsection they also state that CLTC is positively correlated with cytolytic activity. This apparent contradiction requires careful biological reconciliation. I suggest that the authors re examine the gene set used by the ssGSEA

algorithm to define cytolytic activity, as these genes might be confounded by proliferation or metabolic signatures that are upregulated in CLTC high tumors. A proper discussion of this paradox should be added to the Discussion section.

Response: Thanks for your constructive comments. We sincerely thank the reviewer for pointing out this apparent inconsistency and for the constructive suggestion. We carefully re-examined the original correlation analysis results, Figure 6C-D, and the corresponding description in Section 3.3 of the manuscript. After verification, we found that this inconsistency was mainly caused by an incorrect textual description in the original manuscript. The actual results showed that CLTC expression was negatively correlated with both CD8⁺ T-cell infiltration and cytolytic activity. Therefore, these two findings are directionally consistent and do not represent a contradiction. We have corrected this erroneous statement in Section 3.3 of the revised manuscript, and the relevant modifications have been highlighted in red.

(2)The manuscript inconsistently uses the terms “high/low expression groups” and “high/low risk groups” when referring to the same stratification based solely on CLTC median expression (Sections 2.5 and 3.3). Since the grouping does not involve a multi gene Cox model derived risk score, I recommend using “expression groups” uniformly throughout the text to avoid confusion and maintain terminological precision.

Response: Thanks for your constructive comments. We have standardized the relevant wording throughout the manuscript. In the revised version, the term “expression groups” is consistently used to describe the CLTC expression-based grouping, thereby avoiding potential misunderstanding that the grouping was based on a risk-score model.

(3) In Section 3.5, the authors conclude from molecular docking results that 5 fluorouracil and paclitaxel can “effectively bind to CLTC” and imply downstream therapeutic effects. Molecular docking only demonstrates physical binding potential.

Given that CLTC encodes a structural protein (clathrin heavy chain) rather than an enzyme or receptor, the biological consequences of such binding remain speculative. The wording should be toned down to state that these drugs show favorable binding energies in silico, without claiming functional efficacy.

Response: Thanks for your constructive comments. We have revised the relevant statements in Section 3.5, the Discussion, the Abstract, and the Limitations section, with the changes highlighted in red. The original wording, such as “effectively bind to CLTC,” as well as statements implying downstream antitumor effects, has been toned down. The revised text now states that 5-fluorouracil and paclitaxel showed relatively favorable predicted binding energies in the in silico molecular docking analysis, which only suggests the possibility of potential physical interactions with CLTC. We further emphasized in the Discussion that CLTC encodes clathrin heavy chain, which is a structural protein rather than a typical enzyme, receptor, or validated drug target. Therefore, the biological consequences of these predicted binding results remain speculative, and the docking findings cannot be used to demonstrate functional inhibition of CLTC or therapeutic efficacy of these drugs. The revised manuscript now clearly states that the molecular docking results should be regarded as exploratory computational evidence. Further biochemical binding assays and cell-based functional experiments are still required to validate their biological significance and potential therapeutic relevance.

(4)A discrepancy exists between the text in Section 3.2 and Figure 4A regarding the p value for the Kaplan Meier survival analysis in the TCGA cohort. The text reports $p < 0.001$, but the figure legend shows $p = 0.007$. The authors must verify all statistical results and ensure that p values are consistent across the main text, figure panels, and supplementary materials.

Response: Thanks for your constructive comments. We have rechecked the survival analysis results based on CLTC expression grouping in the TCGA cohort and carefully reviewed the main text, Figure 4A, the figure legend, and the related statistical results. We confirmed that the correct P value for the Kaplan – Meier

analysis of the TCGA cohort in Figure 4A is $P = 0.007$. The value reported as $P < 0.001$ in Section 3.2 of the original manuscript was a typographical error, which has now been corrected and highlighted in red. In addition, we have systematically rechecked the other statistical results throughout the manuscript to ensure that all reported values are consistent across different sections.

(5) The nomogram model constructed in Section 3.2 achieves an area under the curve of only 0.66 for predicting 1 year survival in the TCGA cohort. This level of discrimination is modest at best. To improve predictive performance, I suggest performing multivariate Cox regression to identify truly independent prognostic factors among CLTC expression, tumor stage, T category, N category, and other clinical variables. Only those independent factors should be incorporated into a refined nomogram.

Response: Thanks for your constructive comments. We would like to clarify that the primary aim of this section was not to identify independent prognostic factors or to construct an optimized prognostic model based exclusively on independent variables. Rather, our objective was to evaluate the potential clinical prognostic value of CLTC expression in OSCC when combined with conventional clinicopathological indicators. To address this issue, we performed both univariate and multivariate Cox regression analyses. The univariate Cox analysis showed that CLTC expression, age, stage, T category, and N category were associated with overall survival (Figure 4B), whereas in the multivariate analysis, only age and N category remained independent prognostic factors. Because this analysis focused on the integrated clinical value of CLTC expression and clinicopathological indicators, we retained CLTC together with clinically relevant variables that showed prognostic relevance in the univariate Cox analysis in the nomogram model.

(6) The single cell RNA sequencing analysis in Section 2.6.1 mentions that Harmony was used for batch effect correction, but no visualization is provided to show the distribution of samples before and after correction. The authors should include quality control plots, such as t SNE or UMAP projections colored by sample origin, in the

supplementary material to demonstrate that batch effects have been adequately removed.

Response: Thanks for your constructive comments. In the revised manuscript, we have added Supplementary Figure S1 to present the quality-control process for the scRNA-seq data and the results of Harmony-based batch-effect correction. Specifically, Figure S1A shows violin plots of the raw scRNA-seq data from eight OSCC samples before quality-control filtering, including nFeature_RNA, nCount_RNA, percent.mt, and percent.rb. Figure S1B shows the distribution of retained cells after quality-control filtering, indicating that low-quality cells and cells with a high proportion of mitochondrial genes were effectively removed. In response to the reviewer's specific concern regarding batch effects, we added Figure S1C, which shows UMAP plots colored by sample origin before and after Harmony correction. Before correction, cells from different OSCC samples showed a certain degree of sample-dependent separation. After Harmony correction, cells from different sample origins were more thoroughly mixed in the UMAP space, while the overall cellular structure was preserved, indicating that inter-sample batch effects were effectively reduced. We have also added a corresponding description of the quality-control and batch-effect correction procedures in the Results section, clarifying that these steps were completed before subsequent cell clustering, cell annotation, and CLTC expression analysis.

(7)The experimental validation remains largely limited to proliferation and migration assays, yet CLTC's primary physiological role is to mediate clathrin dependent endocytosis. To strengthen the mechanistic link between CLTC expression and tumor biology, the authors can consider to perform an endocytosis related functional assay if conditions permit, for example a transferrin uptake experiment, to confirm that CLTC knockdown genuinely impairs clathrin mediated transport. Such an experiment would directly connect the bioinformatic findings with the biological function of the gene.

Response: Thanks for your constructive comments. We sincerely thank the reviewer for this insightful and constructive suggestion. We fully agree that an

endocytosis-related functional assay, such as a transferrin uptake assay, would provide more direct evidence to link CLTC expression with clathrin-mediated endocytosis in OSCC cells. In the present study, our experimental validation was mainly designed to provide preliminary evidence that CLTC knockdown affects malignant phenotypes of OSCC cells, including proliferation, colony formation, migration, and invasion. Therefore, these assays support the tumor-biological relevance of CLTC, but they do not directly demonstrate impairment of clathrin-mediated transport.

Considering the current scope of this study and the practical limitations during the revision stage, we are unfortunately unable to perform a well-controlled transferrin uptake assay at this time. In addition, we have added this point to the limitation section, stating that future studies should include endocytosis-related functional assays, such as transferrin uptake or receptor internalization assays, to further confirm whether CLTC knockdown directly impairs clathrin-mediated endocytosis and to clarify how this process contributes to OSCC progression.

(8) In Section 3.4, the authors identify a monocyte related gene module (the blue module) using hdWGCNA, but the subsequent functional enrichment analysis of this module reveals pathways such as ribosome and energy metabolism. These are generic features of highly proliferative cells and are not specific to monocytes. To convincingly claim that this module is monocyte associated, the authors should extract and discuss the hub genes within the module that are directly linked to monocyte or macrophage functions, such as polarization markers or cytokine secretion pathways. Without such analysis, the conclusion that the module is specifically related to monocytes appears overstated.

Response: Thanks for your constructive comments. To avoid overinterpretation, we have removed the previous conclusion based on hdWGCNA that the “blue module” was specifically associated with monocytes. The original hdWGCNA-related figures and the corresponding interpretations have been removed from the main figures of the revised manuscript. In the revised manuscript, Section 3.4 has been rewritten. The

focus has shifted from the interpretation of hdWGCNA modules to macrophage-centered ligand-receptor-based cell-cell communication analysis. Specifically, we now show extensive interactions between CLTC-high macrophages and epithelial cells, CAFs, endothelial cells, T cells, inflammatory epithelial cells, and inflammatory macrophages. These interactions are mainly mediated by MIF-, CXCL-, and SPP1-related signaling axes. Therefore, we no longer use ribosome- or energy metabolism-related enrichment results to support monocyte specificity.

Reviewer #4:

The manuscript by Cui et al. investigates the role of CLTC in oral squamous cell carcinoma (OSCC) progression and its association with chemotherapy response. The study employs multi-omics analysis combined with functional experiments to explore the underlying mechanisms. Overall, the study is well-designed and presents interesting findings that contribute to the understanding of CLTC in OSCC pathogenesis. However, there are several aspects that require clarification or improvement before publication. Below are my concerns:

1) The related results indicate that CLTC is upregulated in OSCC and correlated with poor prognosis and chemotherapy resistance. However, the functional validation of CLTC's role in chemotherapy sensitivity remains incomplete. Suggest perform drug sensitivity experiments in OSCC to directly assess the impact of CLTC on chemosensitivity to chemotherapy drugs such as paclitaxel and 5-FU.

Response: Thanks for your constructive comments. We sincerely thank the reviewer for this valuable suggestion. We agree that drug sensitivity assays in OSCC cells would provide more direct evidence to clarify whether CLTC affects the response to chemotherapeutic agents such as paclitaxel and 5-fluorouracil.

In the present study, our analysis of chemotherapy sensitivity was mainly based on public pharmacogenomic data and molecular docking prediction, aiming to preliminarily explore the potential association between CLTC expression and therapeutic response. In addition, our in vitro experiments focused on validating the biological role of CLTC in OSCC malignant phenotypes, including proliferation,

colony formation, migration, and invasion. Therefore, the current results suggest a potential relationship between CLTC expression and chemotherapy sensitivity, but they do not directly establish a causal role of CLTC in regulating the response to paclitaxel or 5-fluorouracil.

Considering the current scope of this study and the practical limitations during the revision stage, we are unable to perform additional drug sensitivity experiments at this time. We have also added this issue to the limitation section, stating that future studies should include drug sensitivity assays, such as CCK-8 or IC50 analyses after CLTC knockdown or overexpression, to directly evaluate the effect of CLTC on the response of OSCC cells to paclitaxel, 5-fluorouracil, and other chemotherapeutic agents.

2) The authors have revealed through bioinformatic analysis that CLTC may be involved in affecting ribosomal function, energy metabolism, and cell adhesion pathways. Have the authors examined the effects of CLTC regulation on key genes in these pathways (e.g., by qPCR)?

Response: Thanks, at present, we have not examined the expression changes of representative genes in these pathways after CLTC knockdown by qPCR. The current experimental validation mainly focused on the protein expression of CLTC in OSCC tissues and cell lines, the efficiency of siRNA-mediated CLTC knockdown, and the effects of CLTC knockdown on the proliferation, migration, and invasion phenotypes of SCC25 cells. In response to your suggestion, we have toned down the relevant mechanistic statements in the revised manuscript. These pathways are now interpreted as bioinformatics-based associations related to CLTC expression, rather than experimentally validated direct regulatory relationships. Meanwhile, we have added statements in the Results, Discussion, Limitations, and Conclusion sections, with the changes highlighted in red, to clarify that future studies should further examine the expression changes of ribosome-related genes, mitochondrial ATP synthesis/oxidative phosphorylation-related genes, and cell adhesion-related genes, such as integrins, cadherins, and collagens, in CLTC knockdown or overexpression models using qPCR,

Western blotting, and functional rescue experiments. These additional experiments will be necessary to verify whether CLTC directly participates in the regulation of these pathways.

3) Given the extensive bioinformatic analyses performed in this paper, the statistical analysis section requires more detailed descriptions: specify the exact statistical tests used for each analysis in the figure legends (e.g., Student's t-test, log-rank test, etc.).

Response: We sincerely thank the reviewer for this valuable suggestion. In response, we have supplemented the figure legends for all figures involving statistical analyses by specifying the exact statistical tests used for each corresponding analysis. All related revisions have been highlighted in red in the revised manuscript.

4) The discussion section should better integrate the findings: (1) Expand on the plausible molecular mechanisms linking CLTC to chemotherapy sensitivity; (2) Compare the results with existing literature and highlight novel contributions; (3) Discuss potential confounding factors and limitations in more detail; (4) Suggest concrete directions for future mechanistic studies.

Response: Thanks for your constructive comments. We have substantially revised the Discussion section to provide a more comprehensive, cautious, and balanced interpretation of our findings. First, we further clarified the novelty of the present study. Although previous studies have reported CLTC-related fusion events in various malignancies, the expression pattern, prognostic value, immune relevance, and functional role of CLTC in OSCC have not been fully elucidated. Therefore, in the revised manuscript, we emphasize that the novelty of this study lies in the integration of bulk transcriptomic cohorts, immune infiltration analysis, single-cell RNA sequencing-based cell-type-resolved analysis, macrophage-centered cell - cell communication analysis, drug sensitivity prediction, molecular docking, and in vitro knockdown validation to systematically evaluate the clinical and biological significance of CLTC in OSCC.

Second, we revised the interpretation of the enrichment analysis results. We now clearly state that enriched pathways, including extracellular matrix organization,

ribosome-related pathways, collagen binding, integrin binding, focal adhesion, platelet activation, and proteoglycan-related pathways, should be interpreted as transcriptome-based pathway-level associations rather than evidence that CLTC directly regulates key genes within these pathways. We also acknowledge that the present study has not validated representative ribosome-related genes, mitochondrial ATP synthesis-related genes, or cell adhesion-related genes by qPCR after CLTC knockdown. To strengthen the Discussion, we added OSCC-related studies supporting the biological importance of extracellular matrix remodeling, ribosomal function, collagen binding, integrin-related signaling, focal adhesion, platelet activation, and proteoglycan-related pathways in OSCC progression.

Third, we expanded the discussion of the immune microenvironment. The revised Discussion further explains that high CLTC expression is associated with reduced CD8⁺ T-cell infiltration, decreased cytotoxic activity, and enhanced immune exclusion-related features, including increased fibroblast- and TGF β -related scores. These findings suggest that CLTC may be associated with immune exclusion and immunosuppressive remodeling of the OSCC tumor microenvironment. We also added studies on CD4⁺ Tfh cells, neutrophil infiltration, PD-1/PD-L1-related signaling, cytolytic activity, and immunotherapy-related immune features in OSCC to better interpret and support our findings.

Fourth, we revised the discussion of drug sensitivity and molecular docking results in a more cautious manner. We now emphasize that the predicted associations between CLTC expression and responses to 5-fluorouracil, docetaxel, paclitaxel, cisplatin, and oxaliplatin should be regarded only as exploratory findings that require further experimental validation. We also clearly state that CLTC encodes a structural protein rather than a classical enzyme or receptor; therefore, the molecular docking results should not be interpreted as evidence of therapeutic efficacy or direct pharmacological inhibition. Instead, we propose several potential mechanisms by which CLTC may be associated with chemotherapy response, including clathrin-mediated receptor trafficking, regulation of EGFR/integrin-related signaling, non-endocytic functions of CLTC in mitotic spindle stability, and its association with

pathway-level changes involving ribosomal function, energy metabolism, extracellular matrix remodeling, and cell adhesion. We also added relevant studies on the use of 5-FU, docetaxel, paclitaxel, cisplatin, and oxaliplatin in OSCC to provide a more clinically relevant interpretation.

Finally, we further expanded the limitations and future directions at the end of the Discussion section. The revised manuscript clearly states that, although the present study systematically evaluated the expression pattern, prognostic value, immune relevance, and potential biological significance of CLTC in OSCC, several limitations remain. First, part of the clinical evidence was derived from retrospective public databases, and the tissue validation cohort was a single-center cohort with a limited sample size, which may affect the generalizability of the findings. Second, prospective multicenter validation and in vivo animal experiments have not yet been performed. Third, although we validated the upregulation of CLTC protein by Western blotting and further confirmed the functional experimental results in a second OSCC cell line, rescue experiments and IHC validation in a larger tissue cohort are still lacking. Therefore, the specificity of CLTC function and its clinicopathological relevance require further confirmation. In addition, we clarified that although enrichment analyses suggested that CLTC may be associated with MAPK, PI3K-Akt, extracellular matrix remodeling, and immune-related pathways, the precise molecular mechanisms remain unclear. The current experiments mainly focused on the malignant phenotypes of OSCC cells and did not directly validate the canonical role of CLTC in clathrin-mediated endocytosis. Moreover, the drug sensitivity prediction and molecular docking results remain computational predictions and correlative findings, which require further experimental validation. All of the above revisions have been added to the Discussion section of the revised manuscript and highlighted in red.