Peer Review File

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Reviewer A

This study is very interesting because it shows the relationship between YAP-1 expression and prognosis in SCLC.

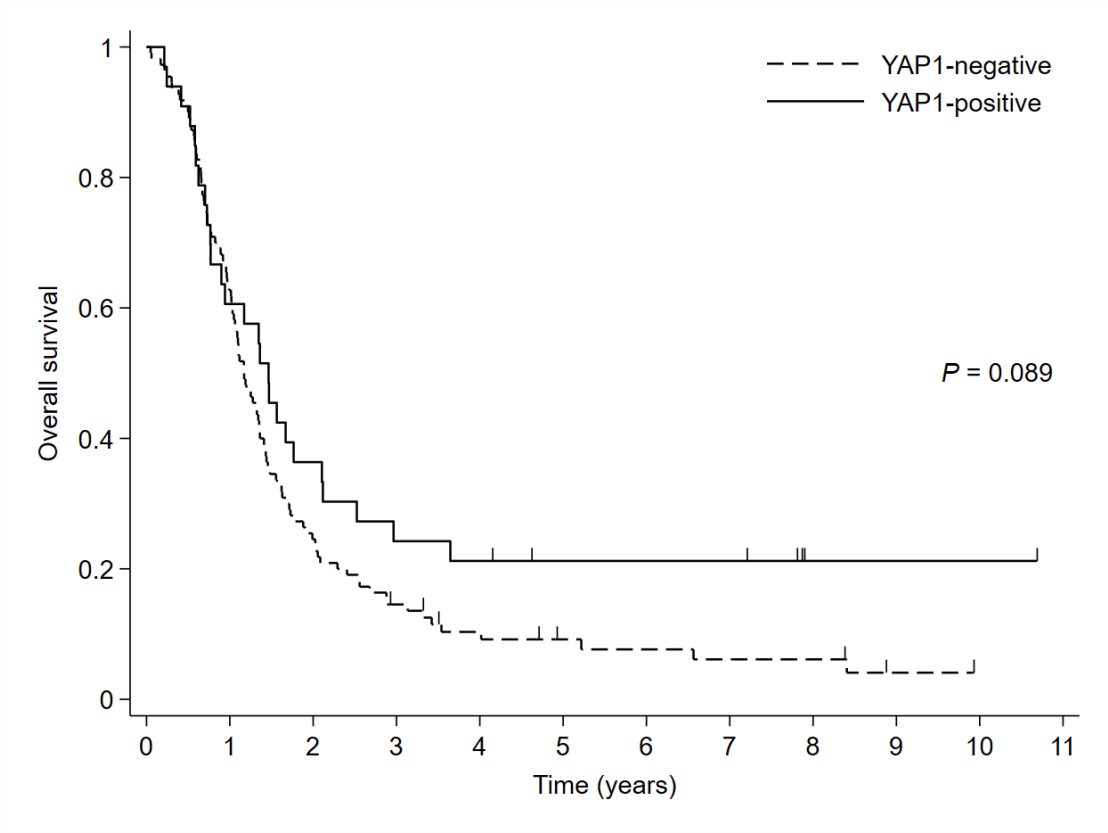
1. YAP1 is expressed in both the nucleus and cytoplasm, but what is the relationship between the expression status of YAP1 in the each cytoplasm and nucleus, and prognosis?

Reply: Thank you for your insightful comment. YAP1 was predominantly expressed in the cytoplasm, with only two cases showing occasional nuclear staining. It appears that nuclear staining of YAP1 is related to the intensity of cytoplasmic staining. In our study, YAP1 scoring was based on cytoplasmic staining. Due to the small number of cases with nuclear expression of YAP1, we were unable to analyze its relationship with prognosis.

Change in the text: (Evaluation of IHC for SCLC subtyping, Methods section) Due to the small number of cases (only two) with YAP1 nuclear staining, we performed the analysis based on cytoplasmic intensity of YAP1 staining only.

2. The cohort is relatively high in patients with poor PS, and there seems to be no statistical difference between YAP-1 positive and negative patients, but the effect on prognosis cannot be ruled out. Would the same trend be seen in PS 0-1?

Reply: Due to the small sample size, particularly in the YAP1-positive group, we did not perform a subgroup analysis beyond tumor stage and NE/non-NE subtype. This limitation has been discussed in the relevant section of our discussion. As shown below, there is a similar trend in the Kaplan-Meier survival curve for the PS 0-1 subgroup. The lack of statistical significance (p=0.089) is likely due to the small sample size (data not included in the revised manuscript).



<Figure: OS according to YAP1-expression in patients with ECOG PS 0-1>

Change in the text: (Limitation, Discussion section) The small subgroup size also made it difficult to statistically confirm the difference in DoR in the LD subgroup that achieved CR (Appendix 5B) and to perform subgroup analysis according to significant clinical variable such as age and ECOG PS.

3. You have mentioned YAP-1 and immune cell infiltration and PD-L1 expression in discussion part. it is recommended that the infiltration of CD8-positive cells and PD-L1 expression status be considered as well in your cohorts. YAP-1 expression in the nucleus has been reported to induce PD-L1 expression.

Reply: Thank you for your suggestion. Unfortunately, we do not have additional whole unstained slides of the cohort, only partial unstained slides remaining after the initial test staining. Since we used small biopsy specimens in this study, many of the paraffin blocks have little material left. As a result, it is challenging to perform additional IHC on this cohort.

Change in the text: (Discussion section) While we have recognized the importance of considering other biomarker expressions such as CD8, PD-L1, and SMARCA4, we were constrained by the availability of our specimens. We had only partial unstained slides remaining after the initial test staining, and many small biopsy specimens had limited material left in the paraffin blocks. Therefore, it was challenging to perform additional IHC on this cohort.

4. you reported the association between YAP-1 positivity and long DoR, but what was the expression status of ASCL1 and NEUROD1 in patients who maintained CR in the YAP-1-positive population?

Reply: Our analysis shows no significant difference in the proportions of ASCL1 and NeuroD1 expression according to CR status within the YAP-1-positive cohort. The distribution of ASCL1 and NeuroD1 expression levels appears similar between patients who maintained CR and those who did not.

Change in the text: (Association of YAP1 expression with treatment response and DoR, Results section) Regarding the expression status of ASCL1 and NeuroD1 in patients who achieved CR within the YAP1-positive group, our analysis showed no significant differences according to CR status (Appendix 6). The distribution of ASCL1 ($p = 0.977$) and NeuroD1 ($p = 0.321$) expression levels was not statistically different between patients who achieved CR and those who did not. These findings suggest that the expression levels of ASCL1 and NeuroD1 may not significantly impact CR status in the YAP1-positive group.

5.In addition to ED and LD classification, staging is generally used. We recommend that staging be included and discussed.

Reply: Thank you for your recommendation. In our cohort, all TNM stage IV patients were classified as ED, and all TNM stage I, II, or III patients were classified as LD, except for two cases with multiple lung nodules (T4N2M0). There were only eight patients with TNM stage I/II. When we analyzed survival excluding TNM stage I/II, stage III had similar results to LD, and stage IV had similar results to ED, as expected. We have added this analysis in Appendix 8 and revised the methods and results sections accordingly.

Change in the text: (Endpoints and assessments, Method section) Baseline characteristics, including age at diagnosis, Eastern Cooperative Oncology Group performance status (ECOG PS), stage (limited-stage [LD] or extensive-stage [ED] according to the Veterans Administration Lung Study Group), TNM stage by the American Joint Committee on Cancer 8th edition, and metastatic lesions at presentation, were collected from electronic medical records.

(Prognostic implications of YAP1 expression on survival outcomes, Results section) Similar patterns were observed in the analyses according to the TNM stage system, with TNM stage III showing results comparable to LD, and TNM stage IV comparable to ED (Appendix 8).

Reviewer B

1. Rather than "molecular subtype", I suggest using the phrase "transcription factor subtype" since the subtype determination is based on immunohistochemistry and not expression profiling.

Reply: Thank you for your suggestion. We have changed the terminology to "transcription factor subtype" throughout the text to accurately reflect the basis of our study.

Change in the text: The term "molecular subtype" has been generally replaced with "transcription factor subtype" throughout the entire manuscript.

2. How many cases of SCLC are pure vs combined? This should be stated and analyzed with regards to the prognostic significance, particularly given that YAP1 expression is known to be associated with combined histology.

Reply: I agree that YAP1 expression may be associated with combined histology. Our study did not include any cases of combined SCLC to maintain a homogeneous cohort. Indeed, combined SCLC has been rarely reported in our institution.

Change in the text: (Patients and study design, Methods section) Patients were excluded from the study if they had a coexisting malignancy or a history of another malignancy within the last five years, experienced histological transformation from non-small cell lung cancer, or had a combined SCLC.

3. ASCL1 and POU2F3 expression is generally believed to be mutually exclusive in SCLC tumor cells, except in exceptional tumors. What the authors report is unusual. It may be helpful to provide images to demonstrate such findings, and to confirm that this is not due to technical artifacts from immunohistochemistry. This should be clarified.

Reply: We appreciate your suggestion. Upon reviewing the ASCL1 IHC results, we found that a case with nonspecific weak diffuse cytoplasmic staining without nuclear staining was incorrectly scored as 1. The intensity score of this case has been revised to 0. After revising the ASCL1 IHC results, only one ASCL1-dominant subtype case showed high POU2F3 expression. This case exhibited tissue necrosis and some cytoplasmic staining in viable tumor cells, likely due to the leakage of nuclear proteins into the cytoplasm caused by cellular damage. The cytoplasmic staining was also observed in tumor cells with squeezing artifacts.

Additionally, Ding et al. reported significant ASCL1 expression in POUF3-dominant SCLC (Ding et al., World Journal of Surgical Oncology, 2022, 20:54, https://doi.org/10.1186/s12957-022-02528-y). Several studies have shown that the molecular classification of SCLC is not entirely exclusive. Our cohort may represent another example of the limitations of this classification system.

Change in the text: (Expression of each transcription factor by dominant subtype, Results section) For example, one ASCL1-dominant SCLC case showed high POU2F3 expression, which exhibited tissue necrosis and some cytoplasmic staining in viable tumor cells, likely due to the leakage of nuclear proteins into the cytoplasm caused by cellular damage (Appendix 3).

Figure 2 and Appendix 2 were slightly modified to reflect the revised expression level of POU2F3.

4. For any p value that is near 0.05 but above (e.g. line 251, p=0.066), they are not significant. The concept of borderline significance is erroneous and should be avoided.

Reply: I absolutely agree with your point and have made the necessary adjustments.

Change in the text: (Association of YAP1 expression with treatment response and DoR, Results section) Notably, when focusing on patients who achieved CR in the LD subgroup, the YAP1-positive group exhibited a substantially longer median DoR compared to the YAP1-negative group (64.8 months [IQR: 46.1–74.8] vs. 36.4 months [IQR: 15.0–41.3]), although the difference was not statistically significant ($p = 0.066$). (Appendix 7).

5. For the YAP1+ group, are they truly SCLC? Based on this recent study (PMID: 38180245), I am worried if the YAP1+ cases here actually represent thoracic SMARCA4-deficient tumors. This should be investigated and clarified.

Reply: The results of the referenced paper are very interesting. However, in actual pathological diagnosis, morphologically non-SCLC tumors are rarely diagnosed as SCLC. The tumor tissues from our cohort are morphologically consistent with SCLC and show no morphological features of SMARCA4-deficient UT, such as vesicular nuclei with prominent nucleoli. Even if SMARCA4 deficiency is present in the tumor morphologically consistent with SCLC, they will be classified as SCLC with SMARCA4 deficiency and not as SMARCA4-deficient undifferentiated tumors.

Change in the text: (Discussion section) Recently, Ng et al. showed that SMARCA4 mutations are commonly observed in SCLC-Y cell lines, leading to reduced SMARCA4 expression and characteristics of SMARCA4-deficient undifferentiated tumors, rather than SCLC. This finding suggests that YAP1 may not be a subtype-determining transcription factor in SCLC (23).

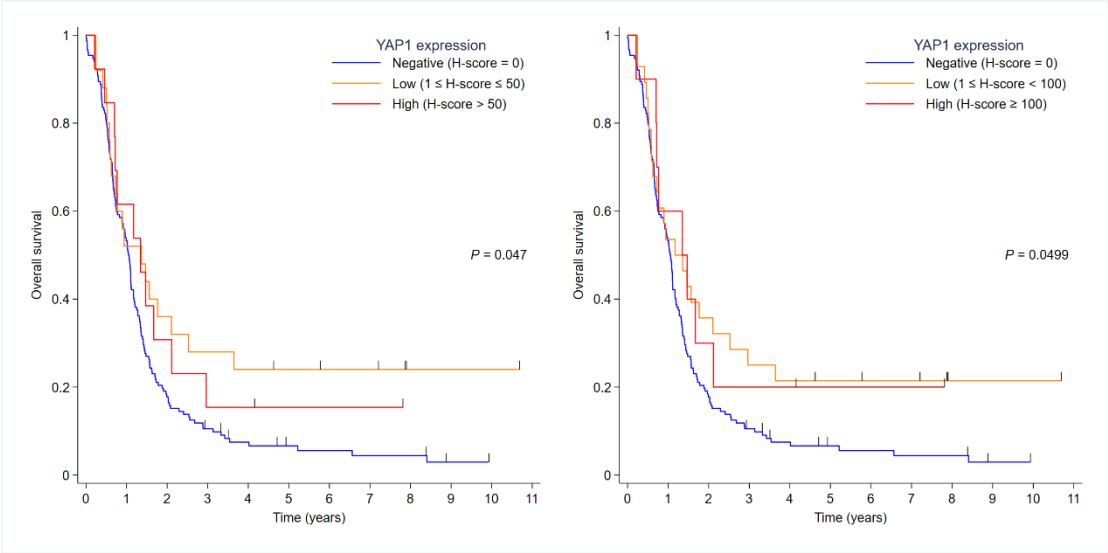
(Discussion section) While we have recognized the importance of considering other biomarker expressions such as CD8, PD-L1, and SMARCA4, we were constrained by the availability of our specimens. We had only partial unstained slides remaining after the initial test staining, and many small biopsy specimens had limited material left in the paraffin blocks. Therefore, it was challenging to perform additional IHC on this cohort.

Reviewer C

In this study on YAP1 expression in SCLC the authors brought up several interesting but also controversial findings. The study is predominantly descriptive and lacks some data about functional aspects.

1. The authors used a Score of 1-50 as low expression and everything above as high. What happens when the high score is shifted to 100 and above?

Reply: When the cut-off for high expression of YAP1 was changed from "above 50" to "100 and above," only 3 out of 13 cases were reclassified from high expression to low expression. This adjustment did not alter the results or their interpretation, as there was no significant difference in overall survival based on the different cut-off values for high expression of YAP1.



Change in the text: None.

2. The authors use the old staging of limited and extensive disease - here the TNM classification should be used. This is mandatory since the 7th classification

Reply: We agree that the TNM classification is necessary when investigating and managing patients with SCLC. In clinical practice, the two-stage classification according to the VA Lung Study Group is still commonly used. We have performed additional analyses using the TNM stage (AJCC eighth edition). All TNM stage IV cases were classified as ED, and all TNM stage I, II, or III cases were classified as LD, except for two cases with multiple lung nodules (T4N2M0). The number of patients with TNM stage I/II was only eight. When we analyzed survival excluding TNM stage I/II, stage III had similar results to LD, and stage IV had similar

results to ED, as expected. We have added this analysis in Appendix 8 and revised the methods and results sections accordingly.

Change in the text: (Endpoints and assessments, Method section) Baseline characteristics, including age at diagnosis, Eastern Cooperative Oncology Group performance status (ECOG PS), stage (limited-stage [LD] or extensive-stage [ED] according to the Veterans Administration Lung Study Group), TNM stage by the American Joint Committee on Cancer 8th edition, and metastatic lesions at presentation, were collected from electronic medical records.

(Prognostic implications of YAP1 expression on survival outcomes, Results section) Similar patterns were observed in the analyses according to the TNM stage system, with TNM stage III showing results comparable to LD, and TNM stage IV comparable to ED (Appendix 8).

3. The authors associate their YAP1 high cases with the inflammatory subtype - this should have been evaluated by IHC and a correlation with YAP1 should be established

Reply: We understand the importance of evaluating the inflammatory subtype by IHC and establishing a correlation with YAP1 expression. However, we do not have additional whole unstained slide sets of the cohort, only partial unstained slides remaining after the initial test staining. Since we used small biopsy specimens in this study, many paraffin blocks have little material left. Therefore, it is challenging to perform additional IHC on this cohort.

Change in the text: (Discussion section) While we have recognized the importance of considering other biomarker expressions such as CD8, PD-L1, and SMARCA4, we were constrained by the availability of our specimens. We had only partial unstained slides remaining after the initial test staining, and many small biopsy specimens had limited material left in the paraffin blocks. Therefore, it was challenging to perform additional IHC on this cohort.

4. As the authors noted a specific association of LD with YAP1, these cases should be classified by TNM; another aspect need to be established: infiltration by immune cells in these low stages

Reply: As described in response to your comment 2, survival curves for stages III and IV were similar to those for LD and ED, respectively. The analysis for stages I/II was not performed due to the small sample size (n = 8). If stages I/II/III were combined into one cohort, the results might be more similar to those of LD. Infiltration by immune cells in low TNM stages could not be evaluated due to limited material remaining in the paraffin blocks. Instead, we have included a plausible explanation in the discussion section of original version manuscript, discussing the potential background supporting the association between LD and immune response, although this remains a hypothesis.

Change in the text: (Prognostic implications of YAP1 expression on survival outcomes,

Results section) Similar patterns were observed in the analyses according to the TNM stage system, with TNM stage III showing results comparable to LD, and TNM stage IV comparable to ED (Appendix 8).

5. OS did not correlate with YAP1; if these cases were staged by TNM and subdivided into oligometastatic disease and widespread metastatic cases, does this show an association to OS?

Reply: In our cohort, YAP1 expression was associated with OS in both univariate and multivariate analyses. As stated, the YAP1-negative group showed worse OS in TNM stages III and IV. When we performed a multivariate Cox regression analysis adjusting for metastatic lesions, YAP1 remained an independent prognostic factor for OS.

Change in the text: (Prognostic implications of YAP1 expression on survival outcomes, Results section) Similar patterns were observed in the analyses according to the TNM stage system, with TNM stage III showing results comparable to LD, and TNM stage IV comparable to ED (Appendix 8).

6. YAP1 is known in association with several molecular abnormalities, and also acts in epithelial-to-mesenchymal transition, the authors should discuss their finding in this respect

Reply: Thank you for highlighting this important aspect. We have added relevant literature to the discussion section.

Change in the text: (Discussion) YAP1 also plays a crucial role in epithelial-to-mesenchymal transition, leading to increased metastatic potential and worse clinical outcomes in various cancers (20-22).

7. Several articles dealing with subtyping of SCLC and the impact of associated oncogenes have been published; YAP1 as a subtype was questioned or excluded based on the data of these studies. A careful search of the literature should be done

Reply: Thank you for this suggestion. To the best of our knowledge, SMARCA4 mutations have been recognized as evidence questioning the appropriateness of YAP1 as a subtype-determining factor. We have revised the discussion section accordingly.

Change in the text: Recently, Ng et al. showed that SMARCA4 mutations are commonly observed in SCLC-Y cell lines, leading to reduced SMARCA4 expression and characteristics of SMARCA4-deficient undifferentiated tumors, rather than SCLC. This finding suggests that YAP1 may not be a subtype-determining transcription factor in SCLC (23).

Reviewer D

The authors performed molecular subtyping of 190 SCLC by IHC and examined the treatment response and overall survival of each molecular subtype. The authors found that the patients with YAP1-positive SCLC tumors had more durable CR and favorable survival compared to those with YAP1-negative tumors, especially in limited-stage. As pointed out by the authors, there are many debates about the role of YAP1 in SCLC, such as the existence of the SCLC-Y subtype and the prognostic values of YAP1. This manuscript did not provide much clarity to these debates due to its retrospective nature, single-institution design, and pre-immunotherapy patient cohort.

1. The percentages of the tumors positive for two subtype markers are surprisingly high. How did the authors validate the antibodies used in this study?

Reply: We validated the antibodies using a home-made tissue block for IHC validation and randomly selected SCLC samples. The validation details are as follows:

	ASCL1	NeuroD1	Pou2F3	YAP1
Positive control tissue	SCLC sample	SCLC sample	Skin (squamous epithelium)	Carcinoma collection block for validation
Negative control tissue	Carcinoma collection block for validation	Carcinoma collection block for validation	Normal tissue collection block for validation	Normal tissue collection block for validation

Ding et al. showed significant ASCL1 expression on POUF3-dominant SCLC. [Ding et al. World Journal of Surgical Oncology (2022) 20:54 https://doi.org/10.1186/s12957-022-02528-y]. To varying degrees, several studies have shown that the molecular classification of SCLC is not entirely exclusive. Our cohort may be another example of the limitations of the classification system.

Change in the text: Appendix 1 was revised.

2. YAP1 is expressed in a high percentage of large cell neuroendocrine tumors, which has a better prognosis than SCLC. Have the tumors of this study been reviewed by a

pathologist specialized in thoracic oncology to confirm the SCLC diagnosis? What percentage of tumors have combined histology of SCLC and NSCLC? Please include the IHC of Ki-67 and NE markers (e.g., chromogranin, synaptophysin, and/or INSM1.

Reply: A pulmonary pathology specialist reviewed the SCLC cases. We collected SCLC cases diagnosed from small biopsy samples. There were no cases of combined SCLC. We have included IHC of NE markers from diagnostic tests, but Ki-67 IHC was not performed at the time of diagnosis.

Change in the text: (Patients and study design, Methods section) Patients were excluded from the study if they had a coexisting malignancy or a history of another malignancy within the last five years, experienced histological transformation from non-small cell lung cancer, or had a combined SCLC.

(Patient and treatment-related characteristics by YAP1 expression, Results section) Although the proportion of strong intensity for IHC markers supporting SCLC diagnosis was generally higher in the YAP1-negative group, only synaptophysin showed a statistically significant difference between the YAP1-negative and YAP1-positive groups ($p = 0.045$).

3. A large part of the discussion was about the immune microenvironment of YAP1-positive SCLC. However, none of these immune cells was examined by IHC in this study.

Reply: We absolutely agree that this is a main limitation of the study. We acknowledge the importance of examining the immune microenvironment through IHC. Unfortunately, we have no additional whole unstained slide sets of the cohort, only partial unstained slides remaining after the initial test staining. Since we used small biopsy specimens in this study, many paraffin blocks have little material left. Therefore, it is challenging to perform additional IHC on this cohort.

Change in the text: (Discussion section) While we have recognized the importance of considering other biomarker expressions such as CD8, PD-L1, and SMARCA4, we were constrained by the availability of our specimens. We had only partial unstained slides remaining after the initial test staining, and many small biopsy specimens had limited material left in the paraffin blocks. Therefore, it was challenging to perform additional IHC on this cohort.

4. In Appendix 6, the patients with extensive-stage YAP1+ SCLC had the same PFS but a better OS than the YAP1- groups. Please provide subsequent treatment information to determine what percentages of patients received immunotherapy after progression from the first-line therapy.

Reply: Thank you for your insightful comment. In Korea, immunotherapy after progression from the first-line therapy had not been approved until the time of analysis. Therefore, there were no cases where patients received immunotherapy after disease progression.

Change in the text: (Patient and treatment-related characteristics by YAP1 expression, Results section) An immunotherapy combined regimen was applied in only 4 patients in total as the first-line therapy, and no patients received immunotherapy after disease progression.

Reviewer E

Interesting study.

Reply: We appreciate your interest in our study and are glad you found it engaging.

Reviewer F

The authors describe a detailed clinicopathological analysis of YAP1 expressing small cell lung cancer (SCLC) and make some important observations about the relationship between YAP1 expression and the favorable survival of patients with SCLC.

YAP1 was initially proposed to define a distinct subgroup; however, it was found to be absent or expressed only at low levels in tumors (PMID: 33482121; PMID: 33011388). Recently, pathogenic mutations in SMARCA4 were identified in six of eight YAP1-expressing SCLC (SCLC-Y) cell lines and correlated with reduced SMARCA4 RNA/protein expression, indicating that the characteristics of SCLC-Y are consistent with SMARCA4-deficient undifferentiated tumors rather than SCLC (PMID: 38180245).

The authors should examine IHC of SMARCA4 to clarify whether SMARCA4 was retained or lost in YAP1 expressing cases in the cohort. It might explain why varied reports show that YAP1 expression was a conflicting survival predictor.

Reply: We appreciate your detailed and insightful comment. The results of the cited studies are very interesting. However, in actual pathological diagnosis, morphologically non-SCLC tumors are rarely diagnosed as SCLC. The tumor tissues from our cohort are morphologically consistent with SCLC and do not show morphological features of SMARCA4-deficient undifferentiated tumors, such as vesicular nuclei with prominent nucleoli. Even if SMARCA4 deficiency is present in a tumor morphologically consistent with SCLC, it would still be classified as SCLC with SMARCA4 deficiency and not as a SMARCA4-deficient undifferentiated tumor.

Additionally, while we recognize the importance of examining SMARCA4 expression in our cohort, we are constrained by the availability of our specimens. We have only partial unstained slides remaining after the initial test staining, and many small biopsy specimens have limited

material left in the paraffin blocks, making it challenging to perform additional IHC on this cohort.

Change in the text: (Discussion section) Recently, Ng et al. showed that SMARCA4 mutations are commonly observed in SCLC-Y cell lines, leading to reduced SMARCA4 expression and characteristics of SMARCA4-deficient undifferentiated tumors, rather than SCLC. This finding suggests that YAP1 may not be a subtype-determining transcription factor in SCLC (23).

(Discussion section) While we have recognized the importance of considering other biomarker expressions such as CD8, PD-L1, and SMARCA4, we were constrained by the availability of our specimens. We had only partial unstained slides remaining after the initial test staining, and many small biopsy specimens had limited material left in the paraffin blocks. Therefore, it was challenging to perform additional IHC on this cohort.