Peer Review File

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Reviewer A

In this manuscript, the authors reported that the CDK4/6 inhibitor abemaciclib, when used in combination with radiation therapy and PD-1 inhibitors, is effective against Rb-deficient small cell lung cancer (SCLC) and exhibits immunostimulatory effects involving activation of the STING pathway. However, the study fails to conduct any experiments testing whether the immunostimulatory effects mediated through the STING pathway would be canceled by inhibiting this pathway. Therefore, the true immunostimulatory effects mediated by the STING pathway and the involvement of CD8-positive cells in exerting anti-tumor effects remain unclear, representing significant limitations. Furthermore, it is practically impossible to concurrently administer CDK4/6 inhibitors, PD-1 inhibitors, and radiation therapy for advanced SCLC cases, which poses a notable challenge both clinically and experimentally.

Reply:
Thank you for your insightful comments on our manuscript. Actually, a considerable amount of research has demonstrated the association between STING pathway activation and anti-tumor immunity currently. Among them are studies conducted in SCLC. Lauren A. Byers' team in 2019 first demonstrated in SCLC models that the immunostimulatory effect following DDR inhibition originates from the activation of the STING-TBK1-IRF3 pathway and the increased release of downstream chemokines CCL5 and CXCL10, which were validated by knockdown experiments targeting CGAS, STING, and IRF3 (1). Conghua Xie's team indirectly proved the primary role of CD8+ T cells in STING-mediated immunostimulatory effects by depleting CD8+ T cells using anti-CD8 antibodies (2).

However, we have noticed that we lack relevant quotes for this part in the article, and have supplemented them in the main text. Additionally, we recognized that although these studies serve as reference points, the wording "via STING pathway activation" in the title and conclusion sections is not rigorous without validation through knockout experiments. We noticed that some literature (2,3) similarly illustrates the induction of anti-tumor immunity by referencing previous studies and detecting activation of the STING pathway, although knockout experiments have not been conducted for validation. They do not directly emphasize "via STING activation" in the title or conclusion but rather use phrases like "inflame/modulate the TME" etc. Consequently, we have made appropriate modifications according to relevant studies.

Certainly, the initiation of any preclinical research should be aimed at serving the purpose of clinical practice. Based on our previous study (4), the MATCH trial (NCT04622228) is ongoing, using EP chemotherapy combined with LDRT and atezolizumab to treat patients with advanced ES-SCLC. The preliminary data of this study indicate that the toxic reactions are tolerable. For SCLC patients, chemotherapy resistance is almost inevitable, so our study may provide some options for salvage therapy. Since we have demonstrated the safety of LDRT+ICI in clinical patients, the

efficacy and toxic side effects of combining low-dose CDK4/6 inhibitor abemaciclib with this regimen remain unknown. However, based on our study, we insist that abemaciclib+LDRT+ICI is logically coherent and has certain exploratory value.

Once again, thank you for your suggestions and invaluable feedback. Your expert opinions have played a crucial role in enhancing the quality of our research. We appreciate your time and look forward to your response.

Changes in the text:
We added some relevant quotes about the association between STING pathway activation and anti-tumor immunity:

- Page 13, line 436-438.

We replaced the wording "via STING pathway activation" to “by inflaming the tumor microenvironment”, and modified some expression in abstract, introduction and conclusion:

- Page 1, line 4-5;
- Page 2, line 40-47;
- Page 4, line 134-139;
- Page 17, line 563-567.

References

1. Sen T, Rodriguez BL, Chen L, et al. Targeting DNA Damage Response Promotes Antitumor Immunity through STING-Mediated T-cell Activation in Small Cell Lung Cancer. Cancer Discov 2019;9:646-61.
2. Zhang N, Gao Y, Huang Z, et al. PARP inhibitor plus radiotherapy reshapes an inflamed tumor microenvironment that sensitizes small cell lung cancer to the anti-PD-1 immunotherapy. Cancer Lett 2022;545:215852.
3. Sen T, Della Corte CM, Milutinovic S, et al. Combination Treatment of the Oral CHK1 Inhibitor, SRA737, and Low-Dose Gemcitabine Enhances the Effect of Programmed Death Ligand 1 Blockade by Modulating the Immune Microenvironment in SCLC. J Thorac Oncol 2019;14:2152-63.
4. Wang H, Yu M, Na F, et al. Striking effect of low-dose radiotherapy combined with PD-1 blockade on small cell lung cancer in mice and refractory patients (Achilles Study). Journal of Clinical Oncology 2022;40:e20608-e.

Reviewer B

Summary
This manuscript investigates the efficacy and mechanism of action of CDK4/6 inhibition in Rb-deficient SCLC. This manuscript addresses a critical unmet need for patients with SCLC by identifying potential therapeutic strategies that may have activity in these difficult to treat tumor types. The authors hypothesized that a therapeutic combination of CDK4/6 inhibition plus anti-PD-1 antibody plus low-dose radiation that may slow tumor growth and harness an immune-mediated response.

Major concerns

There are several conflicting data points and lack of clarity noted in the manuscript:

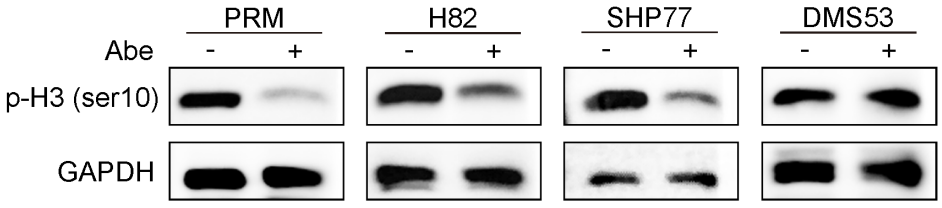
1. Using Rb-deficient cell lines, the authors demonstrate that G2/M arrest is induced in response to abemaciclib; however, the Western blot data shows decreased expression of the 'markers' they are using for G2/M checkpoint including pCDK1 and PLK1. I would also argue that more accepted protein expression markers of G2/M arrest can include pH3 (ser10), Chk1, etc. In addition, did the authors determine if G2/M arrest is sustained and leads to apoptosis? Or is there a population of cells that escapes arrest? A time course experiment with assessment of apoptosis (i.e., cleaved PARP-1) could clarify.

Reply 1:

We appreciate your thorough examination. Our data showed that abemaciclib induced G2/M cell arrest in Rb-deficient SCLC cells, which is not contradictory to the downregulated G2/M checkpoint actually. In conclusion, abemaciclib induced downregulation of G2/M checkpoint, leading more SCLC cells entering into the M phase prematurely, which is also manifested as an increase in the proportion of G2/M phase cells on a macroscopic level.

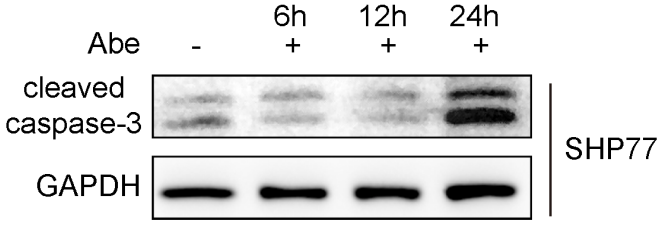
However, an increased proportion of SCLC cells entering into M phase does not mean that SCLC cells acquire stronger proliferative ability. To understand this, we first need to grasp the unique characteristics of SCLC cells themselves. SCLC is a tumor with highly unstable genomes, and maintaining the integrity of DNA during replication is a crucial task for them. Cell cycle checkpoints are indispensable for monitoring DNA damage and subsequent DNA repair. Due to prevalent mutations of *RB1* and *TP53*, the G1 checkpoint almost loses its function in SCLC, making the function of G2/M checkpoint vital for DNA damage repair and genome stability in SCLC cells. If the G2/M checkpoint in SCLC cells is inhibited, they will enter the M phase with damaged and unrepaired DNA. When damaged DNA accumulates to a certain extent, cells are unable to undergo mitosis, and may even leading to apoptosis, which was so called as mitotic catastrophe (1).

Therefore, inhibiting the G2/M checkpoint in SCLC would lead to premature entering into the M phase with unrepaired DNA, promoting apoptosis or inducing ds-DNA to activate STING pathway. This principle has been applied in the development of novel therapies. As a specific example, the team led by Triparna Sen utilized an inhibitor of WEE1, a G2/M checkpoint regulator, to treat SCLC cells. WEE1 inhibition led to G2/M arrest, downregulation of p-CDK1 (Tyr15) expression, and increased proportion of apoptosis in SCLC cells (2).



Upon reviewing our data, both the downregulation of p-CDK1 (Tyr15) expression and the GSEA analysis indicating downregulation of the G2/M checkpoint pathway align with the aforementioned viewpoints. Additionally, as advised, we examined the alteration of p-H3 (ser10) expression after treating four SCLC cell lines with abemaciclib for 24 hours. Theoretically, chromatin condensation occurs as cells enter the M phase, forming chromosomes, and histones, represented by histone H3,

undergo phosphorylation. However, our results revealed a significant downregulation of p-H3 (ser10) expression in three Rb-deficient SCLC cell lines. We propose the following interpretation: the decreased expression of p-H3 (ser10) in SCLC cells entering into M phase indirectly indicates that mitosis did not proceed smoothly. Considering that we have already demonstrated the downregulation of PLK1 after abemaciclib treatment, which would also lead to mitosis inhibition, these results corroborate each other. Finally, we believe that for SCLC cells, when there is dysfunction in G2/M checkpoint, the cells may be in an "intermediate state" where they cannot effectively repair damaged DNA or proceed smoothly through mitosis. Therefore, using "G2 phase" or "M phase" alone to describe this state may not be entirely accurate.



In summary, our data indicate that abemaciclib downregulates the G2/M checkpoint in SCLC cells, resulting in premature entry into the M phase. Furthermore, our study demonstrated that abemaciclib can induce increased ds-DNA damage in SCLC cells. Therefore, we believe that the combined effects of G2/M arrest and ds-DNA damage induction may lead to more SCLC cells undergoing apoptosis. As we reported in Fig 1G, after 24 hours of abemaciclib treatment, not only did it induce G2/M arrest in three Rb-deficient SCLC cell lines, but it also promoted apoptosis in them. As advised, we examined the alteration of cleaved caspase-3 expression in SHP77 cells following abemaciclib treatment for 6 h, 12 h, and 24 h. The results showed that the expression of cleaved caspase-3 increased significantly at 24 h, indicating the occurrence of apoptosis. It should be emphasized that there is a certain causal relationship between cell cycle arrest, DNA damage accumulation and apoptosis. We did not clearly elucidate the specific molecular mechanisms and potential sequence of events by which abemaciclib induces DNA damage, cell cycle arrest, and apoptosis in SCLC cells, and this is one of the limitations of our study.

Changes in the text:

We added some quotes in the results to help understanding this:

- Page 11, line 342 and 358-360.

2. Similarly, the gene expression data is also conflicting with the induced G2/M arrest in response to abemaciclib. What would be the mechanism of decreased gene expression in response to CDK4/6 inhibition?

Reply 2:

Questions related to the downregulation of the G2/M checkpoint gene expression have been addressed in Reply 1. In another study, abemaciclib also downregulated the expression of the G2/M checkpoint in breast tumors (3).

In addition to Rb protein, CDK4 and CDK6 also regulate numerous other downstream targets. Therefore, the biological effects of CDK4/6 inhibition are extensive and complex, and the effects after inhibition are also different for different cell types (4).

Changes in the text:

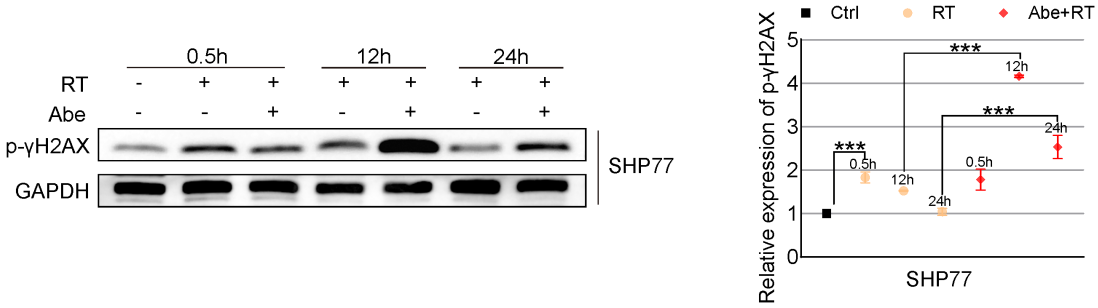
The same as Reply 1, we added some quotes in the results to help understanding this:

- Page 11, line 342 and 358-360.

3.In figure 2 the authors claim there was decreased DDR, however only showed decreased expression of a few proteins. A decrease in DDR would need to be shown by inducing DNA damage followed by time to recovery/resolution of DNA damage (presence or absence).

Reply 3:

Thank you for your insightful comments on our manuscript. Indeed, it would be inaccurate to claim "induced DDR inhibition" without directly demonstrating that abemaciclib can decelerate the rate of DNA repair. It's worth noting that while some studies have demonstrated that CDK4/6 inhibition leads to DDR inhibition and consequently promotes ds-DNA damage, other studies have also indicated that CDK4/6 inhibition can directly induce ds-DNA damage by enhancing the generation of reactive oxygen species (ROS) (5,6). Therefore, DDR inhibition and DNA damage generation after CDK4/6 inhibition may represent two independent processes in some cases. So, we have revised almost all instances of the term "induced DDR inhibition" in the article to the more accurate "induced increased ds-DNA damage."



In addition, we used SHP77 cells to assess the alteration in p-γH2AX expression following radiotherapy alone or in combination with abemaciclib for 0.5 hours, 12 hours, and 24 hours. We observed that the expression of p-γH2AX peaked immediately after radiotherapy and gradually declined to the level of the control group over time, indicating a gradual repair of DNA damage. However, when combined with abemaciclib, we observed a significant upregulation of p-γH2AX expression at 12 hours, surpassing the peak expression level observed with radiotherapy alone. This suggests that abemaciclib may not only simply delay the repair of DNA damage following radiotherapy but also increase the generation of DNA damage through other way. This also supports our viewpoints above.

Changes in the text:

We replaced almost all the expression “induced DDR inhibition” to “induced increased ds-DNA damage” :

- Page 1, line 34;
- Page 2, line 41, 48 and 59;
- Page 4, line 134-135;
- Page 12, line 377-381;
- Page 13, line 412-414, 426;

- Page 16, line 513-515;
- Page 17, line 554, 563-564.

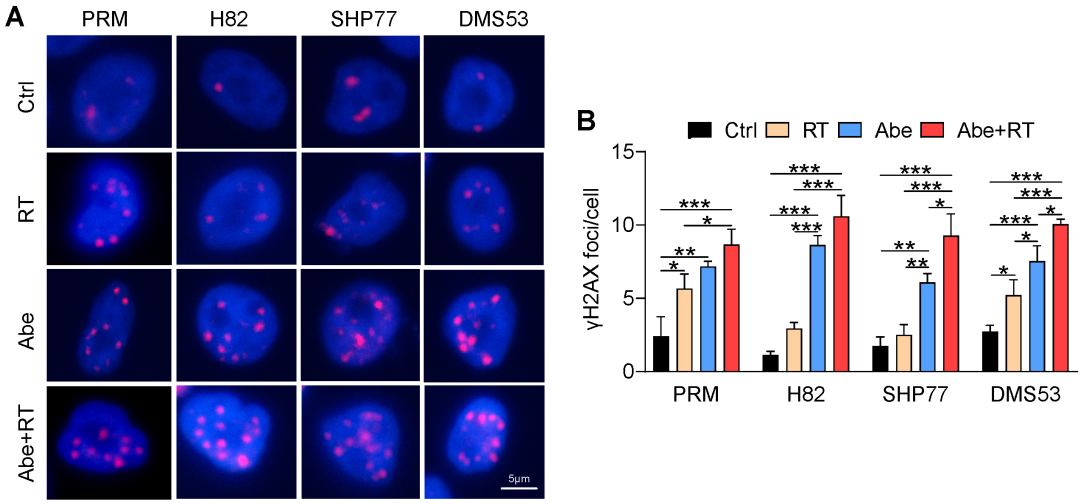
We also added some quotes about the Rb-independent manner to induce DNA damage through ROS:

- Page 17, line 506-510.

4.For figure 2C, it is not clear why the 'ds-DNA' is mostly outside the DAPI stained DNA? Could also use anti-γH2AX antibody here.

Reply 4:

Thank you for your attention and guidance. "ds-DNA" means double-strand DNA. In the nucleus, if damage-induced DNA double-strand breaks cannot be repaired in time, some of them will enter the cytoplasm in the form of fragmented ds-DNA through nuclear pores. In addition, mitochondrial damage caused by oxidative stress will also lead to the release of ds-DNA from the mitochondria. That's why ds-DNA is mostly outside the nucleus. In our study, we utilized anti-ds-DNA antibody to stain SCLC cells, aiming to directly demonstrate the presence of cytosolic ds-DNA. Team of Conghua Xie also used anti-ds-DNA antibody to stain cytosolic ds-DNA in SCLC cells (7).



As advised, we also utilized anti-p-γH2AX antibody for staining and observed that treatment with abemaciclib for 24 hours induced more γH2AX foci formation in all four SCLC cell lines, consistent with our Western blot results. γH2AX appears only at sites of DNA damage repair, thus primarily localizing in the nucleus. In contrast, free ds-DNA can enter into the cytoplasm through nuclear pores, so it is mainly concentrated in the periphery of the nucleus and the cytoplasm, which is the difference between the two staining.

Changes in the text:

As advised, we added the IF staining results in the results and Supplementary Fig 3:

- Page 12, line 394-396;
- Page 28, line 836-844.

We also added some content to describe the difference between γH2AX and ds-DNA:

- Page 12, line 396-399.

5. The authors then jump to activation of the STING pathway without confirming presence of cytosolic DNA. While the increase in downstream cytokines/chemokines is relevant to changes in the immune landscape, the authors did not demonstrate a clear mechanism for this nor did they mention any potential mechanisms in the discussion.

Reply 5:

We appreciate your insightful comments. As addressed in Reply 4, the purpose of our ds-DNA immunofluorescence staining was precisely to demonstrate the increased cytosolic ds-DNA.

Currently, a considerable amount of research has demonstrated the association between STING pathway activation and anti-tumor immunity. Among them are studies conducted in SCLC. Lauren A. Byers' team in 2019 first demonstrated in SCLC models that the immunostimulatory effect following DDR inhibition originates from the activation of the STING-TBK1-IRF3 pathway and the increased release of downstream chemokines CCL5 and CXCL10, which were validated by knockdown experiments targeting CGAS, STING, and IRF3 (8). Conghua Xie's team indirectly proved the primary role of $CD8^+$ T cells in STING-mediated immunostimulatory effects by depleting $CD8^+$ T cells using anti-CD8 antibodies (7). We have noticed that we lack relevant quotes for this part in the article, and have supplemented them in the main text. Additionally, we recognized that although these studies serve as reference points, the phrase "via STING pathway activation" in the title and conclusion sections is not rigorous without validation through knockout experiments. Consequently, we have made appropriate modifications.

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- Page 1, line 4-5;
- Page 2, line 40-47;
- Page 4, line 134-139;
- Page 17, line 563-567.

Minor points:

1. The use of ‘ds-DNA’ is used frequently. Please define. Is this referring to DNA damage? DNA double-strand breaks?

Reply : We have defined the wording “ds-DNA” in Page 1, line 34 and Page 4, line 134. In this text, “ds-DNA” alone means fragmented double-strand DNA; “ds-DNA damage” means DNA damage leading to fragmented double-strand DNA formation.

2. The name of the anti-PD-1 antibody used was not included in Methods.

Reply : We have added the CatalogNumber of anti-PD-1 antibody in Page 5, line 157.

We would like to express our sincere gratitude for your thorough review and valuable suggestions

on our submitted manuscript. Your professional knowledge and patient guidance have provided us with valuable insights, enabling us to better understand and articulate our research. Once again, thank you for your suggestions and invaluable feedback. We appreciate your time and look forward to your response.

References

1. Rudin CM, Brambilla E, Faivre-Finn C, et al. Small-cell lung cancer. Nat Rev Dis Primers 2021;7:3.
2. Taniguchi H, Caeser R, Chavan SS, et al. WEE1 inhibition enhances the antitumor immune response to PD-L1 blockade by the concomitant activation of STING and STAT1 pathways in SCLC. Cell Rep 2022;39:110814.
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4. Goel S, Bergholz JS, Zhao JJ. Targeting CDK4 and CDK6 in cancer. Nat Rev Cancer 2022;22:356-72.
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6. Wang H, Nicolay BN, Chick JM, et al. The metabolic function of cyclin D3–CDK6 kinase in cancer cell survival. Nature 2017;546:426-30.
7. Zhang N, Gao Y, Huang Z, et al. PARP inhibitor plus radiotherapy reshapes an inflamed tumor microenvironment that sensitizes small cell lung cancer to the anti-PD-1 immunotherapy. Cancer Lett 2022;545:215852.
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