Peer Review File

Article information: https://dx.doi.org/10.21037/tlcr-23-567

Reply to Reviewer A

Dear Reviewer,

Thank you very much for your time involved in reviewing the manuscript and your very encouraging comments on the merits.

Comment 1: A great deal of caution must be taken when the fundamental aspects of any experimental protocol involve the over expression of a particular gene in tissue culture and in an organoid. There is no doubt that tumor cells, particularly in the acidic environment in which they live, highly regulate the expression of genes and the authors have created an artificial environment to achieve the goals of their research. It's a dangerous approach since it is highly likely that levels achieved by the authors will never be reflected in the tumor. The world has moved on from simple overexpression experiments and now genetic manipulation provides a more reliable outcome. I could live with this overexpression approach if the authors would clone a truncated version of the protein containing several domains that upon disruption will disable the function of the protein and demonstrate that not simply the overexpression of any fragment of this protein but the complete protein, results in the results they present. Frankly, by using a genetic approach where the gene is disabled or activated or slightly in- activated and long-term cultures will result in a more sustainable conclusion.

Reply 1: We thank the reviewer for the comment and the reviewer's comment is greatly appreciated. In our study, we established the PFKP overexpression clone and the results showed that PFKP overexpression increased the resistance ability of NSCLC cells to CDDP. Furthermore, we established the PFKP shRNA stable cell lines by using shRNA sequences which was conformed to downregulate the mRNA expression of PFKP and the cells were selected for 10 days with 0.5 μg/ml puromycin for 48 h after infection. Genetic inhibition of PFKP enhanced CDDP sensitivity both *in vitro* and *in vivo*. The level of PFKP overexpression or silencing was frequently measured in the *in vitro* assay in the study to conform the effect of upregulation or downregulation expression of PFKP by western blot assay. Certainly, we agree with the reviewer's comment that it would be better to use more stable assay such as clone truncated, or CRISPR-Cas9 approach to construction of PFKP stable cell lines. This is indeed a part that we need to strengthen in future research.

Comment 2: It's also important to note that NRF2 is the key player to drug resistance in lung cancer and is upstream from the genes in which they choose to manipulate. So, the status of master regulators are much more important than these authors seem to realize.

Reply 2: We thank the reviewer for the comment and the reviewer's comment is greatly appreciated. It is reported that the importance of NRF2 in drug resistance is closely linked to its control on iron metabolism as a transcription factor. In fact, NRF2 has a key role in the prevention of lung cancer cells from ferroptosis, a cell death mechanism involving iron-dependent lipid peroxidation. Therefore, it would be interesting to examine if NRF2 is required for PFKP-mediated cellular drug resistance in lung cancer.

Comment 3: There is no doubt that it is a struggle to develop lung cancer models in mice. The authors inject the standard cell line A549s in nude mice. The problem is that it is not in the microenvironment of the lung, and I particularly worried about the repetitiveness of the injections… If I am reading this correctly, between lines 302 and 305, illustrated in Figure 3A, experimental protocols involve injection every three days up to 42 days when the mice were sacrificed according to the authors. This seems like a forced series of experiments trying to make the result they want for their expectations, dangerous approach.

Reply 3: We thank the reviewer for the comment and the reviewer's comment is greatly appreciated. In order to better mimic the microenvironment of lung, we will further explore the biological function and molecular mechanism of PFKP-mediated tumor cell resistance using a lung in situ injection model in the future. The experimental procedure: Cut open the right skin and muscles, and you can see the fluctuating lung lobes with breathing. Inject a 100ul cell mixture (cell suspension and matrix glue mixed in a 1:1 ratio) vertically into the middle lobe of the right lung, and then suture and then disinfect.

In addition, our experimental protocols involve injection with CDDP (a first-line drug for lung cancer) every three days up to 42 days when the mice were sacrificed. This procedure is based on relevant literature [1,2].

References:

1. Tyagi A, Kaushal K, Chandrasekaran AP, Sarodaya N, Das S, Park C-H, Hong S- H, Kim K-S, Ramakrishna S: CRISPR/Cas9-based genome-wide screening for deubiquitinase subfamily identifies USP1 regulating MAST1-driven cisplatin- resistance in cancer cells. Theranostics 2022, 12(13):5949-5970.

2. Teng JP, Yang ZY, Zhu YM, Ni D, Zhu ZJ, Li XQ: Gemcitabine and cisplatin for treatment of lung cancer in vitro and vivo. Eur Rev Med Pharmacol Sci 2018, 22(12):3819-3825.

Comment 4: I recommend a major revision where the authors increase their literature and background to include current methods being tried to reduce chemoresistance in lung cancer and frankly, other solid tumors. While the references in total look appropriate, I would suggest focusing more on technologies and why their approach will provide new information. And that's the second point, I'm not sure this paper provides any new information especially with the level of dosing and sensitivity that needs to be required for translational activity. Seems a bit out of the mission of this journal and so, the authors need to focus much more attention on establishing why this information is more than a series of experiments to show what we already know. Start by saying what the actual discovery is state more clearly how this work will lead to a translational module and go from there.

Reply 4: We thank the reviewer for the positive comment on our manuscript. As requested by the reviewer, Firstly, we have carefully edited the entire manuscript and had the revised manuscript polished by professional editors to be more concise before resubmission. In our study, we elucidated the underlying mechanism by which PFKP mediates chemoresistance in tumor tissues. Specifically, PFKP enhanced the expression of ABCC2 through increasing the activity of NF-κB. The findings of this study enhance our comprehension of the molecular mechanisms associated with chemoresistance in NSCLC and may have significant implications for the development of more efficacious therapeutic interventions for this pathology. As suggest by the reviewer, the background and references about the role of PFKP include cellular drug resistance in lung cancer has been incorporated into the revised manuscript.

Reply to Reviewer B

Dear Reviewer,

Thank you very much for your time involved in reviewing the manuscript and your very encouraging comments on the merits.

Comment 1: There is no indication in the section relating to Clinical Specimens as to the number of NSCLC patient tissues used in the study. Furthermore, there is no table presented showing patient demographics & characteristics. These are standard requirements when publishing data using patient material.

Reply 1: Thank you for your suggestion. We appreciate your feedback and understand the importance of including patient demographics and characteristics in the publication. In response to your comment, we added a supplementary table labeled “Supplementary Table 1” specifically dedicated to presenting the patient demographics and characteristics. This will ensure that all necessary information is provided for readers to better understand the study population. We thank you for bringing this to our attention and we will make the appropriate changes in the revised version of the paper.

Changes in the text: See the uploaded annex "Supplementary Table 1" for details.

Comment 2: The methods are written in the present tense instead of past tense.

Reply 2: We do appreciate the reviewer`s comments and we are sorry for the mistake in this manuscript. As requested by the reviewer, we have carefully edited the methods of manuscript by using the past tense before resubmission.

Changes in the text: The modifications are mainly in the "**Construction of lung cancer organoids**" section of the **Methods**.

Comment 3: Where centrifugation speeds are alluded to in this section, (eg. 100g), this should be represented as 100 x g.

Reply 3: We thank the reviewer for the comment and the reviewer’s comment is greatly appreciated. As requested by the reviewer, centrifugation speeds (eg. 100g) have been changed to 100 × g.

Changes in the text: The tube was inverted on a single-cell suspension preparation system, with a temperature set at 38.5℃ and speed set at 100 × g, and it was rotated clockwise and counterclockwise for 5 minutes.

Comment 4: While the study focuses on cisplatin, the authors allude to the use of paclitaxel at one point yet there are not substantial data presented showing the use of this drug across experiments.

Reply 4: We thank the reviewer for the comment and the reviewer's comment is greatly appreciated. Cisplatin and paclitaxel are the basic drugs for clinical chemotherapy of lung cancer. In our study we demonstrated that overexpression of PFKP was correlated with poorer survival rates in NSCLC patients who received platinum-based chemotherapy. Using NSCLC organoid, we found that the expression of PFKP was elevated in cisplatin-resistant patients with NSCLC. Overexpression of PFKP decreased the sensitivity of NSCLC cells to cisplatin, while genetic inhibition of PFKP enhanced cisplatin sensitivity both *in vitro* and *in vivo*. Although our study did not elucidate the biological function and molecular mechanism of PFKP and paclitaxel resistance in lung cancer, however, we found that NSCLC samples with high PFKP expression exhibit variable degrees of resistance to conventional chemotherapeutic agents such as cisplatin and paclitaxel by using the organoid experiments. Therefore, further elucidating the molecular mechanism of PFKP gene involvement in paclitaxel resistance in lung cancer is of great significance.

Comment 5: How as the A549-PFKP-ABCC2-siRNA cell line generated? Not mentioned in this section.

Reply 5: We do appreciate the reviewer`s comments and we are sorry for the confuse in this manuscript. A549-PFKP-ABCC2-siRNA refers to knockdown of ABCC2 in A549-PFKP stable cell lines. Firstly, transfection of PFKP plasmids was performed using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. A549-PFKP stable cell lines expressing PFKPwere selected for 10 days with 0.5μg/ml puromycin 48 h after infection. Secondly, ABCC2- siRNA was transfection into A549-PFKP stable cell lines by using the Lipofectamine 3000 reagent.

Changes in the text: The following section has been added to the Methods:

Prior to siRNA transfection, A549-PFKP stable cell lines, which had been pre- screened with puromycin, were treated with trypsin and incubated overnight to reach a fusion rate of 60-70%. ABCC2-siRNA was then transfected into the A549-PFKP cells using lipofectamine 3000 (Thermo Fisher, L3000015).

Comment 6: For Western blot analyses, what dilution of secondary antibodies were used?

Reply 6: We do appreciate the reviewer`s comments and we are sorry that we did not write these points clearly in our originally submitted manuscript. The dilution of secondary antibodies has been incorporated into the revised manuscript.

Changes in the text: Then, after washing, the appropriate secondary antibodies were used based on the primary antibodies. HRP-conjugated rabbit anti-goat IgG (1:3000) and HRP-conjugated goat anti-mouse IgG (1:5000) were applied and incubated for 1 hour at room temperature.

Comment 7: How was the cDNA synthesized in the section on qRT-PCR? The authors only mention that "cDNA was amplified using an ABI 7500 qPCR instrument". This is insufficient detail. In addition, there is no detail provided as to the amplification conditions used in running mRNA analyses on the ABI platform for PFKP, ABCC2 and GAPDH.

Reply 7: Thank you for your comments and we apologize for the lack of detail provided in the original draft. For cDNA synthesis we used the PrimeScript ™ RT reagent Kit (Takara, Japan, No. RR047A), and the conditions used were strictly in accordance with the manufacturer's instructions, and the reverse transcription conditions were as follows: 37℃ 15 min, 85℃ 5 sec, 4℃ ∞. Similarly, for qPCR we used TB Green® Premix Ex Taq™ II (Takara, Japan, No. RR820L) and performed the experiment according to the instructions as follows:

Holding Stage

Step1: 95℃ for 30 sec Cycling

Stage

Number of Cycles: 40

Step1: 95℃ 5 sec Step2:

60℃ 30 sec Melt Curve

Stage

In the manuscript, we have added the relevant product item numbers in order to minimize confusion for the reader.

Changes in the text: The Trizol method was used to isolate total RNA from the cultured cells. The PrimeScript ™ RT reagent Kit (Takara, Japan, No. RR047A) and PCR equipment (Bio-Rad, USA) were used for cDNA synthesis. The cDNA was amplified using an ABI 7500 real-time PCR instrument (Applied, USA) and TB Green® Premix Ex Taq™ II (Takara, Japan, No. RR820L).

Comment 8: Some minor grammatical errors throughout manuscript. In some sections, CDDP is written as "DDP". Under the in vivo mouse experiments, the concentration of cisplatin administered i.p. is not appropriately expressed as mg/kg in this section. Yet, in the results section, it is given as 2mg/kg. How were mice sacrificed prior to harvesting of tumours? Not sure whether the additional information provided on the animal laboratory at the end of this methodology section is necessary, unless requested specifically as part of the journal guidelines.

Reply 8: Thank you for your careful review. The misrepresentation of cisplatin (CDDP) in the manuscript has been revised. In the section "**Animal experiments**" the dosage used for CDDP was incorrectly stated as "0.002mg/10g", which has been revised to 2mg/kg. For the humane execution of experimental mouse, we employed
an intraperitoneal injection of 0.3% sodium pentobarbital followed by cervical dislocation. This method ensures that the mice are first rendered unconscious by the anesthetic before being euthanized swiftly and painlessly through cervical dislocation. It is worth noting that this approach aligns with established ethical standards for animal research. To ensure transparency and compliance with ethical guidelines, we have also included the ethical approval certificate for the animal experiments. This certificate serves as evidence that the experimental procedures were conducted in accordance with the applicable ethical standards for animal welfare.

Changes in the text:

line 207: A549/CDDP (sh-control and shRNA#1) line

209: CDDP (2mg/kg, CDDP/animal weight)

Comment 9: No information provided on primary or secondary antibody concentrations used in IHC analyses. Were slides counter-stained and if so, how was this done?

Reply 9: Thanks for your great suggestion on improving the accessibility of our manuscript. The following concentrations were used for primary antibodies: PFKP (1:800), survivin (1:800), ABCC2 (1:500), p65 (1:2000), and 1:500 for secondary antibodies.We have made additional corrections in the corresponding sections of the manuscript. For statistical analysis of the IHC images, the Color Deconvolution tool in ImageJ software was utilized to determine the percentage of DAB stained areas. Subsequently, Prism software was employed for further statistical analysis.

Changes in the text: In the "**Immunohistochemistry (IHC) and Immunofluorescence Analysis**" section. The appropriate primary antibodies were added individually in drops according to the dilution ratios in the antibody instructions and being kept at 4°C overnight, appropriate secondary antibodies were applied. The following concentrations were used for primary antibodies: PFKP (1:800), survivin (1:800), ABCC2 (1:500), p65 (1:2000), and 1:500 for secondary antibodies.

Comment 10: When describing the TUNEL assay, more detail on cell seeding densities would be useful.

Reply 10: We do appreciate the reviewer`s comments and we are sorry that we did not write these points clearly in our originally submitted manuscript. The detail on cell seeding densities of TUNEL assay has been incorporated into the revised manuscript. Changes in the text: 5×10^4 cells were inoculated and cultured overnight on cover slip and were subject to a TUNEL assay, and an in-situ cell death detection kit was used for this purpose. Images of the samples were taken through AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss).

Relevant content has been added to the "**Methods**" section.

Comment 11: In Fig. 1B, what are the differences between the survival plots shown for overall survival? These are not clearly defined.

Reply 11: We do appreciate the reviewer`s comments and we are sorry that we did not write these points clearly in our originally submitted manuscript. The overall survival analysis In Fig. 1B was performed in the public database Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service&cancer=lung). The analysis data shows that high PFKP expression was not only associated with worse overall survival in the cohort of CaArray database, but also in all the dataset which including more than 17 cohorts' data. The above descriptions have added to the manuscript for better understanding.

Changes in the text: For lung cancer patients receiving chemotherapy across CaArray dataset, high expression of PFKP was associated with poorer overall survival (OS) and post-progression survival (PPS) compared to those with low expression.

Similarly, in the GSE14814 database, high PFKP expression was significantly correlated with poorer OS. The combined analysis of more than 17 cohorts' data also revealed a significant correlation between high PFKP expression and poorer OS and first-progression survival (FPS) (Fig. 1B).

Comment 12: Fig. 1C is poorly explained.

Reply 12: Thank you for the suggestions you have made. The results shown in Figure 1C indicate that PFKP expression is positively correlated with CDDP resistance in both datasets, as evidenced by the two graphs on the left. However, the two panels on the right show that there was no statistically significant correlation between PFKP expression and the efficacy of the two drugs, methotrexate and fluorouracil. These findings set the stage for further research into the factors associated with PFKP that contribute to CDDP resistance. This important aspect will be discussed in subsequent sections in order to fully analyze the underlying mechanisms of CDDP resistance. We have added to the descriptions for better understanding.

Changes in the text: The GSEA analysis revealed that PFKP overexpression was closely associated with CDDP chemotherapy markers, but not found in methotrexate and fluorouracil sets, indicating that PFKP expression may contribute to CDDP resistance in NSCLC

Comment 13: Supplmentary Fig. 1 is poorly presented. Based on the experimental analyses associated with this figure, it does not represent with scientific accuracy and justification, the findings described. How can one tell from this figure that these organoids from various NSCLC patient samples are chemoresistant when no cisplatin dose-response or treatment with vs no treatment controls are shown?

Reply 13: Thank you for the additional information. Supplementary Figure 1 depicts an image that show the fully constructed organoid, highlighting its intricate and detailed structure. This figure emphasizes the successful completion of the organoid construction process and serves as visual evidence of the achievement. After organoid establishment, we treated the organoids with a series of drug concentration gradients, and then detected the ATP activity and calculated the IC50 value, as described in "In vitro drug sensitivity screening". We added the results of the pharmacological sensitivity experiments to Supplementary Figure 1B.

Changes in the text: Please see the uploaded annex "Supplementary Figure 1B" for details.

Comment 14: In Fig. 1D, there does not appear to be appropriate controls shown here in this Western blot. While this does show expression in response to cisplatin (+),

there are no untreated (-) controls shown for each sample. In light of this, it is not possible to deduce changes in expression of PFKP based on what is shown here. **Reply 14:** Thank you for clarifying the details. In Figure 1D, a Western blot (WB) experiment was conducted using proteins extracted from a clinical sample. The "-" refers to a sample that exhibited sensitivity to CDDP treatment, based on the drug sensitivity results obtained from the organoid assay. Conversely, the "+" represents a sample that demonstrated resistance to CDDP treatment. The sample on the right side of the figure represents the CDDP-resistant A549 cells that were constructed for comparative analysis. The main objective of this figure is to highlight the differential upregulation of PFKP expression in CDDP-resistant patients, which closely resembles the expression observed in the A549 cell line.

Changes in the text: We apologize for the confusion and have removed the "-" and "+" labels.

Comment 15: Fig. 1E, more appropriate labelling of panels required. Are these representative of NSCLC tumours pre-treatment vs those from the same patient following the development of cisplatin resistance?

Reply 15: Thank you for your patience in reviewing this. In Fig. 1E, the immunohistochemistry (IHC) images also originate from clinical samples. A representative image was selected from each of the two categories: chemo-sensitive and chemo-resistant. The images are labeled with sample numbers 011 and 007, respectively. It's important to note that these samples were not taken before and after treatment. Instead, the samples included in this study were obtained from patients who underwent direct surgical treatment.By including these representative IHC images, the study aims to visually demonstrate the differential staining patterns
between chemo-sensitive and chemo-resistant samples, providing further evidence for the association between PFKP expression and CDDP resistance in these patients.

Changes in the text: We displayed two representative images, and to reduce confusion, we have retained one of the images every group.

Comment 16: Fig. 1F shows graphical data for "% area of PFKP". No clear as to what this means. What method of scoring was used here to quantify PFKP protein expression in these clinical tissue samples? From the images shown, it is clear that staining is higher in chemo-resistant tumours. However, in the accompanying graph, this shows the opposite. This is also contradictory to what is stated in the results section for this figure where it states that "...expression of PFKP was significantly elevated in chemo-resistant organoids compared with the chemo-sensitive samples".

Reply 16: Thank you for your careful review and pointing out the errors in our work. For statistical analysis of the IHC images, we used the colour deconvolution tool in the ImageJ software to determine the percentage of DAB-stained positive areas in the images, which were then further statistically analysed using the Prism software, and are therefore described as "% area of PFKP". The significance of immunohistochemistry is also corroborated by the results of WB in Figure 1D, which demonstrates that the expression of PFKP is increased in chemotherapy-resistant patients, and that an error in labelling was made in the original submission, which has now been corrected.

Changes in the text: Please see the revised Figure 1.

Comment 17: While quantitative data is shown for mRNA expression in Fig. 2B, there is no graphical data shown for protein in Fig. C.

Reply 17: Thank you for your comment. We have performed quantitative analysis of the Western blot (WB) results of Figure 2C in the revised Figure 2.

Changes in the text: Please see the revised Figure 2.

Comment 18: In Fig. E, "fold-change in colony number" is shown. How were colonies measured/quantified? For Figs. D and G (Western blots), how many experimental replicates were carried out here.

Reply 18: Thank you for your comment. In Figure 2E, the “fold-change in colony number” was determined by quantifying the number of colonies using ImageJ software. Statistical analysis and the subsequent generation of the graph were performed using Prism. For Figures 2D and 2G (Western blots), the experiment was independently replicated three times. This means that the WB experiments were repeated three times to ensure the reliability and reproducibility of the results. We have added the descriptions in the figure legends.

Comment 19: When referring to the colony formation assay, the authors refer to "tumour cell proliferation". While this is a clonogenic assay, it would be more accurate to use the terminology "cell surviving fractions".

Reply 19: Thank you for your comment and suggestion. We agree and have made the necessary revisions in the text.

Changes in the text: The results demonstrated that overexpression of PFKP increased cell surviving fractions (Fig. 2E) and decreased CDDP-induced cell apoptosis (Fig. 2F) in A549 cells.

Comment 20: In Fig. 3 (in vivo), the labelling of this figure showing mice/tumours, would indicate that all groups were treated with cisplatin which adds some confusion to this. Is this correct? In the same section (Fig. 3C), the figure legend refers to n=5 mice per group, which is different to what is stated in the materials and methods section of n=6 mice/group. In the figure legend for Fig. 3C, it states that "mice were sacrificed 24hr after last administration". However, in the materials and methods section, it refers to..."one day 42, tumours were removed". This is inconsistent and difficult to follow with these contradictions in information.

Reply 20: Thank you for bringing these concerns to our attention and providing clarification. We apologize for any confusion caused by the inconsistencies in the figure labeling and the discrepancy in the number of mice stated in the figure legend compared to the materials and methods section. Regarding Fig. 3C, where it is mentioned that n=5 mice per group in the figure legend, this was indeed a typographical error. The correct number should be n=6 mice per group, as stated in the materials and methods section. We appreciate your carefully reading, and we have corrected this error in the figure legend. Furthermore, we apologize for the inconsistency in the timing of sacrifice mentioned in the figure legend and the materials and methods section. To clarify, the mice were sacrificed 24 hours after the last administration (day 41) of the drug, which refers to day 42 in the experimental timeline. This aligns with the information provided earlier in the text.

Changes in the text: Figure 3. **PFKP promotes chemoresistance of NSCLC tumors in vivo.** (A, B) Four groups (n = 6) of BALB/c nude mice inoculated with the different cells were sacrificed on day 42, and tumor tissue was harvested. The weight and size of the tumors were measured. (C) HE staining was performed on mouse tumor tissues, and the expression of survivin was detected using IHC. HE,hematoxylin-eosin; IHC, immunohistochemistry. * $p < 0.05$.

Comment 21: All figure legends are lacking in information on the statistical test used.

Reply 21: Thank you for your feedback. We apologize for the oversight in not including information about the statistical tests used in the figure legends. It is important to provide this information for transparency and reproducibility. To address this concern, we have revised all the figure legends to include a clear statement about the statistical tests employed for data analysis. By doing so, we provided essential information regarding the statistical analyses performed for each figure in the figure legends.

Changes in the text: See "**Legends** " in the manuscript.

Comment 22: In the results section relating to ABCC2 expression, data are shown for "intracellular CDDP content". This is confusing as this would imply measuring the intracellular levels of cisplatin within cells as measured by mass spectrophotometry such as ICP-MS. It is not clear from these data whether this is intracellular/total proteins?

Reply 22: We do appreciate the reviewer`s comments and we are sorry that we did not write these points clearly in our originally submitted manuscript. In order to detect intracellular CDDP content, we collected the indicated cells in the log phase of growth were suspended in medium at a density of 1×10^6/ml and incubated for 4 h in the presence of CDDP at 37 °C in a humidified atmosphere containing 5% CO_2. The intracellular platinum level was determined by atomic absorption spectrophotometry.

At the same time, the total cell protein was measured and the CDDP content in the total protein was analyzed.

Changes in the text: The above description has been added to the **Methods** section of the manuscript.

Comment 23: How was DNA-bound cisplatin measured?

Reply 23: I'm very sorry to have been a nuisance to you. Similar to the previous question, we have added a description of this experimental method in the **Methods** section.

Comment 24: In Fig. 4C, the data shown in graphs for A549-vector vs A549-PFKP do not reflect the image shown for same. A more accurate representative image is required that corresponds to the quantitative data presented.

Reply 24: Thank you for pointing out the discrepancy between the representative image and the quantitative data presented in Fig. 4C. We apologize for any confusion caused by this inconsistency. We understand the importance of providing accurate and representative images that correspond to the quantitative data. In light of your comment, we included the quantitative data in the revised version of Fig. 4C.

Comment 25: Fig. 4I, the authors refer to "monoclonal formation". In the context of the data shown, it is not clear what this means.

Reply 25: Thank you for providing additional clarification regarding Fig. 4I. We corrected "monoclonal formation" to “clone formation”.

Comment 26: The figure legend for Fig. 4 needs amending, in particular, that relating to "five cells". This should be five cell types or similar phrasing of same.

Reply 26: Thank you. We amended the figure legend for Fig. 4.

Changes in the text: (C) Immunofluorescence staining of the five cell types after treatment with 5 μg/mL of CDDP for 48h. Antibodies against γ-H2AX (Ser139) (red), as well as Hoechst 33342 (blue; nuclei), were used. The results represent the mean ± SEM of five independent experiments.

Comment 27: Where cell/tissue images are shown, there is no magnification mentioned.

Reply 27: Thank you for pointing this out. We added the magnification in the image caption or legend.

Changes in the text: See "**Legends** " in the manuscript.

Comment 28: The authors allude to IkBalpha-mutant A549 cells, yet there is not indication of these genetically manipulated cells or how this mutant cell type was generated in the materials and methods section of the manuscript.

Reply 28: We do appreciate the reviewer`s comments and we are sorry that we did not write these points clearly in our originally submitted manuscript. The pBabe- Puro-IκBα-mut (plasmid 15291) expressing mutant IκBα was from Addgene (Cambridge, MA).

IkBalpha-mutant A549 cells was obtained by transfection of IκBα-mut plasmids was performed using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction.

Changes in the text: The above description has been added to the **Methods** section of manuscript.

Comment 29: In Fig. 6, panels should be labelled as PFKP low and PFKP high. While survival data are presented from online datasets using Kaplan Meier plotter analysis, there are no survival data shown for the NSCLC patient tissues stained as part of this study. This is a major weakness in this section of the manuscript on clinical relevance.

Reply 29: Thank you for providing additional information about Figure 6 and the limitations of the clinical samples in our study. In Figure 6 case1 is a patient with high expression of PFKP and case2 is a patient with low expression of PFKP. We have labelled the panels as PFKP low and PFKP high. We also acknowledge that the clinical samples used in the study were collected at the end of 2021, and due to the limited follow-up period and small sample size, complete long-term follow-up data was not available for inclusion in the manuscript. In light of these constraints, it is important to emphasize the preliminary nature of the findings and acknowledge the need for further validation with larger clinical samples. Prominently stating the limitations in the manuscript and discussing the potential impact of a larger sample size and extended follow-up would help to address the concerns raised regarding the clinical relevance of the findings. Thank you for providing these clarifications. If you require further assistance or have any more questions, please feel free to ask.

Changes in the text: (A) The expression of ABCC2 and p65 in clinical samples with high (case1) versus low (case2) PFKP expression were examined separately using IHC.