Peer Review File

Article information: https://dx.doi.org/10.21037/tlcr-24-160

Reviewer A

The manuscripts focus on the RASA2 negatively regulates p53 in cancer cells and promotes radioresistance, providing a new predictive biomarker and a potential therapeutic target for radioresistance. I think this article is very interesting and important for Oncology research, especially lung cancer. However, some parts of the article need to be modified.

As you proceed with the manuscript, consider the following suggestions:

1. Consider further discussing the role of RASA2 expression in gender and stage of lung cancer patients in the manuscript.

Reply: *Thanks for the review pointing out here, we discussed the discrepancy of RASA2 expression in gender and stage in the discussion section (Page 14, line 480-489)*

Changes in the text:

Based on TCGA datasets of Lung Adenocarcinoma (LUAD) and Lung Squamous Cell Carcinoma (LUSC), it was observed that there are no significant differences in the expression levels of RASA2 between male and female lung cancer patients. This finding is particularly intriguing as it suggests that the expression of RASA2 in lung cancer is likely not influenced by hormonal differences between genders, such as those attributed to estrogen and testosterone. Typically, hormones play significant roles in the progression and development of lung cancer, and their lack of influence on RASA2 expression warrants further investigation. In addition, we found no statistical significance in RASA2 expression levels across the various stages of lung cancer. This uniformity in expression levels from early to advanced stages of the disease suggests that RASA2's expression is consistent throughout the progression of lung cancer. This consistency underscores the potential importance of early detection of RASA2 expression in patients.

2. In the context of radiotherapy, how does the phenomenon of RASA2 inhibitor correlate with acquired radiosensitive to radiotherapy and the associated epigenetic factors (If available)?

Reply: *That's a good question. Unfortunately, there is no commercially available RASA2 inhibitors. We would expect the future RASA2 inhibitors can recapitulate most of those finding in RASA2 deficient cells, but also the inhibitors may have more potent antitumor efficacy by targeting both tumor cells and immune*

cells. In support of this, the recent paper (PMID:36002574) showing RASA2 ablation in T cells boosts antigen sensitivity and long-term function.

3. Consider providing more details on radiation dose of 8 Gy, why SF of RASA2 in lung cancer cells with GOF-mutant p53 is equal or no different compared with control? In addition, how do these changes contribute to the regulation of gene expression at the molecular level?

Reply: *Thanks, our data suggested that RASA2 played a negative role to p53 when the function of p53 is still intact. In other words, if p53 is loss or mutated, RASA2 may loss the specific interaction with p53 and modulate p53 expression at the molecular level. We also revised this in the result section. (Page10,line349-361)*

Changes in the text:

In our investigation into the potential impact of RASA2 on cell lines featuring gain-of-function (GOF) characteristics resulting from p53 mutations, specifically p53 175H or p53 273H, commonly observed in various human cancers (17). To simulate these conditions, we introduced the aforementioned p53 mutants into our HCT 116 p53(-/-) cancer cell line (Supplementary Figure 1A). It's worth noting that the HCT 116 p53(-/-) cell line lacks endogenous p53 expression, a crucial factor in ensuring that any discernible effects attributed to the mutant p53 variants would not be influenced by dominant-negative inhibition of wild- type p53. Contrary to our initial expectations, the inhibition of RASA2 did not yield the anticipated restoration of radiosensitivity in our experimental setup, as evidenced by the assessment of colony formation (Supplementary Figure 1B). Additionally, RASA2 inhibition did not result in a significant increase in apoptosis, as shown in Supplementary Figure 1C. These unexpected findings suggest a nuanced relationship between RASA2 and p53 functionality. Notably, it appears that RASA2 may predominantly exert its influence in a negative manner when the function of p53 is intact. The lack of observed radiosensitivity restoration and apoptosis induction in the presence of p53 mutants raises intriguing questions about the specific interplay between RASA2 and p53, shedding light on potential complexities in their functional relationship within the cellular context. Further exploration is warranted to unravel the underlying molecular mechanisms governing these interactions and to decipher the intricacies of RASA2 involvement in the context of p53-mutant cell lines.

4. Please discuss more about the connection of your results of RASA2 expression in cell line, animal, and human. Any limitation? In addition, please provide example to support your discussion.

Reply: *We have revised the discussion part of the limitation and provided an example to support. (Page 11, line462-475)*

Changes in the text:

Initially, it's important to highlight that RASA2 frequently co-occurs with mutations in other tumor suppressors, such as Neurofibromatosis type 1 (NF1). For example, in melanoma, loss of RASA2 and NF1 have been found to have complementary pro-tumorigenic functions (PMID: 30478445). This co-mutation suggests that focusing solely on RASA2 might not be as effective, due to the interconnected nature of these pathways, potentially limiting the broader applicability of our findings. Another limitation arises from the methodology used to study the RASA2 mutation profile. By relying on primer sequences from pre-existing research, our approach may not capture the complete mutation landscape of RASA2. This reliance potentially limits the accuracy and comprehensiveness of our findings, leaving the true mutation frequency and spectrum of RASA2 only partially understood. Advancements in sequencing technologies and more bespoke primer design could alleviate this issue, offering deeper insights into RASA2's role in cancer. Lastly, the exclusion of the immune system's role from our study represents a significant oversight, given the growing recognition of immuno-oncology in cancer therapy. Specifically, the potential synergies between immune checkpoint inhibitors and RASA2 mutations were not explored. This area of research could unveil novel therapeutic strategies by modulating immune response to fight cancer more effectively.

Reviewer B

The authors investigated that RASA2 involved in radiosensitivity of patients with non-small cell lung cancer. Certainly, highly RASA2 mRNA expression was negatively correlated to radiosensitivity and also prognosis. In in vitro experiments, RASA2 knockdown using siRNA induced more DNA damage as well as apoptotic cells. These in vitro experiments were performed using p53 wild-type NSCLC cell lines, so there are some concerns as to whether it is cancer-specific.

- p53 is a gene that mutates frequently in patients with lung cancer. Therefore, the mainstream treatment is to restore p53 function. RASA2 function should be investigated when p53 is mutated.
Reply: *We did some investigation, please see supplementary Figure 1. We firstly introduce p53 mutants p53 175H or p53 273H in our HCT116 p53(-/-) cancer cell line. In this case, we found RASA2 inhibition failed to restore radiosensitivity or inducing apoptosis, suggesting, a nuanced relationship between RASA2 and p53 functionality.*

- Indeed, you use a p53-/- cell line, HCT115, which is a colorectal cancer cell line. Why did you use HCT116?

Reply: *Good point. The reason is p53 proficient and deficient HCT116 cell line is commercially available, and we used this cell line as a tool to investigate the interaction between RASA2 and p53 at molecular level, meanwhile bypass the confounding variations across different cell lines.*

- Did you perform experiments using RASA2-specific inhibitors in in vivo studies? There is no finding of whether it is cancer-specific.

Reply: *Unfortunately, there is no commercially available RASA2 inhibitors. We would expect the future RASA2 inhibitors can recapitulate most of those finding in RASA2 deficient cells, but also the inhibitors may have more potent antitumor efficacy by targeting both tumor cells and immune cells. In support of this, the recent paper (PMID:36002574) showing RASA2 ablation in T cells boosts antigen sensitivity and long-term function.*

Specific-comments

- Please explain plasmid experiments briefly in Materials and Methods.

Reply: *Thanks, we have provided more details about plasmid experiment*. (Page5, line 149-167).

Changes in the text:

To introduce the 175H and 273H mutations into the p53 pCB6+ constructs, we performed site-directed mutagenesis by using NEB's Q5® Site-Directed Mutagenesis Kit (NEB #E0554) according to the manufacture instructions. The following oligonucleotides were used for 175H: forward primer: AGC GAG GTT GTG AGG CAC TGC CCC CAC CAT GAG CGC TGC CCC CAC CAT GAG CGC TGC T; reverse primer: AGC AGC GCT CAT GGT GGG GGC AGT GCC TCA CAA CCT CCG T. Meanwhile, the following oligonucleotides were used for 273H: forward primer: GGA ACA GCT TTG AGG TGC CATG TTT GTG CCT GTC CTG G; reverse primer: CCA GGA CAG GCA CAA ACA TGC ACC TCA AAG CTG TTC C. Briefly, we first performed exponential amplification to get the PCR product, then PCR product was treated with Kinase, Ligase &DpnI (KLD) reaction for up to 1 hour at room temperature. 5ul of KLD MIX was transformed into 50ul competent cells (Takara, Cat#636763), incubated on ice for 30 minutes, then we performed heat shock at 42°C for 30 seconds, expanded the cells by adding 950 µl SOC, gently shake at 37°C for 1 hour. 250 µl of SOC media was spread onto ampicillin agar plate (100 µg/mL), incubated overnight at 37°C. The single colony was picked up the next day and performed miniprep using QIAprep Spin Miniprep Kit according to manufacture instructions (QIAGEN, cat# 27104). Then the vector was sent to sequence.

For generation cell lines with gain-of-function (GOF)-mutant p53, an empty vector of pCB6+ or constructs expressing p53 175H or 273H (in the PCB6+ vector with the 72R polymorphism) were used and transfected

with effectene reagent (QIAGEN, cat#301425). Subsequently, cells were selected using G418 (600 μg/mL). The efficacy was confirmed by western blot.

- Please describe the details of irradiation condition i.e., geometry and linac detail.
Reply: *Thanks, we have provided more details about irradiation condition.(Page3, line100)*
Changes in the text:
In addition, we stratified patients into a s sensitive group [complete response (CR) or partial response (PR)] and an insensitive group [stable disease (SD) or progressive disease PD)] 1 month after CyberKnife treatment（non-isocentric，6-megavolt (MV) linear accelerator）

- In animal experiments, please describe the effects of treatment regimen. 10 Gy/day is the regimen for stereotactic radiotherapy, but it is not implemented for stageIIIa NSCLC.
Reply: *That's a great question. For the orthotopic lung cancer mice model, we used 24 Gy of irradiation (1.6 Gy/day x15 days) to better recapture similar treatment option in stageIIIa NSCLC patients, aiming to maximize tumor control while minimizing toxicity. For localized irradiation in subcutaneous tumor model (HCT116), we performed 10Gy/day, which is more standard treatment regimen based on previous literature (https://www.sciencedirect.com/science/article/pii/S1350448721001475) with treatment efficacy and cost effective.*

- P8 L254, "come types" to "some types"?
Reply: *Thanks, we already corrected the typo error in the manuscript.(Page 9,line302)*
Changes in the text: *some types*

Reviewer C

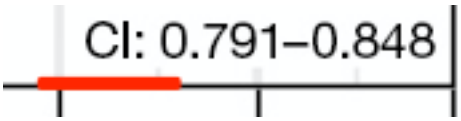
1. Please indicate the source of mice (bought from where).

238 ##Animal experiments ↵

239 Six-week-old male BALB/c nude mice were kept in a pathogen-free environment.
240 performed under a project license (No. 143890054R) granted by ethics board of Huaz

Reply: *Those mice were purchased from BEIJING HFK BIOSCIENCE CO.,LTD. We have revised it.*

2. Figure 1
a. Please revise it to "95% CI" in figure 1B.



Reply: *we have revised it.*

b. Since figure 1E is obtained from HPA database, please follow the policy from The Human Protein Atlas database (link: https://www.proteinatlas.org/about/licence). Please provide the URL that links directly point to cell map, not the homepage of website. Otherwise, we suggest removing Figure 1E.

550 400×). (E) RASA2 staining in tissue microarrays of lung cancer from The Human Protein Atlas (Images
551 are available from v23.0.proteinatlas.org). (F) The whiskers of box plots of the radiotherapy response

Reply: *We have provided the URL link.*

Here is an example:

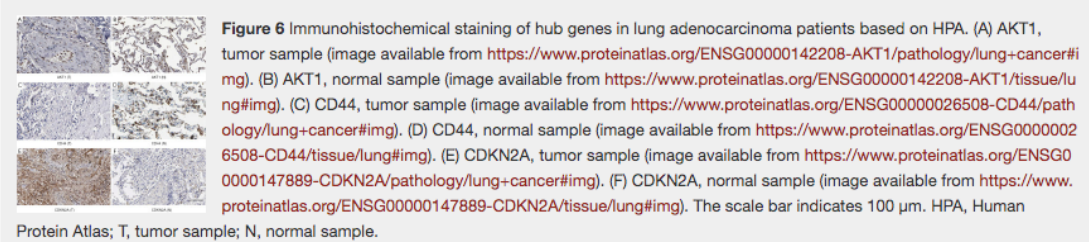
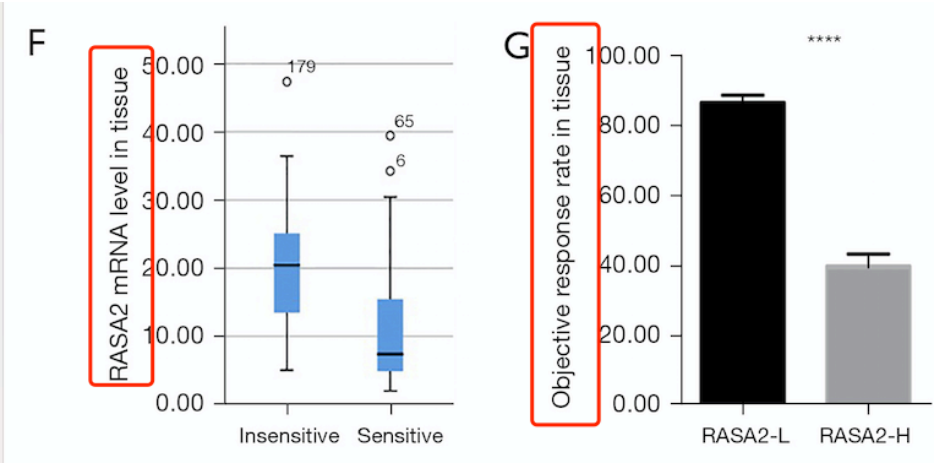


Figure 6 Immunohistochemical staining of hub genes in lung adenocarcinoma patients based on HPA. (A) AKT1, tumor sample (image available from https://www.proteinatlas.org/ENSG00000142208-AKT1/pathology/lung+cancer#img). (B) AKT1, normal sample (image available from https://www.proteinatlas.org/ENSG00000142208-AKT1/tissue/lung#img). (C) CD44, tumor sample (image available from https://www.proteinatlas.org/ENSG00000026508-CD44/pathology/lung+cancer#img). (D) CD44, normal sample (image available from https://www.proteinatlas.org/ENSG00000026508-CD44/tissue/lung#img). (E) CDKN2A, tumor sample (image available from https://www.proteinatlas.org/ENSG00000147889-CDKN2A/pathology/lung+cancer#img). (F) CDKN2A, normal sample (image available from https://www.proteinatlas.org/ENSG00000147889-CDKN2A/tissue/lung#img). The scale bar indicates 100 μm. HPA, Human Protein Atlas; T, tumor sample; N, normal sample.

c. Please also indicate the staining method (or observation method) and magnification of figure 1E in the legend.

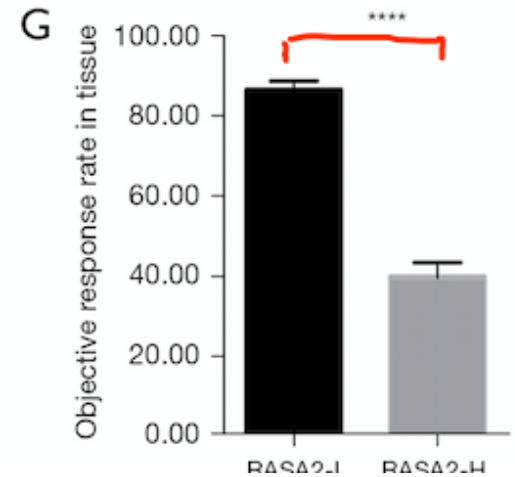
Reply: *Thanks, we have added it.*

d. If applicable, please indicate the unit of the Y-axis.



Reply: Thanks, we have indicated the unit.

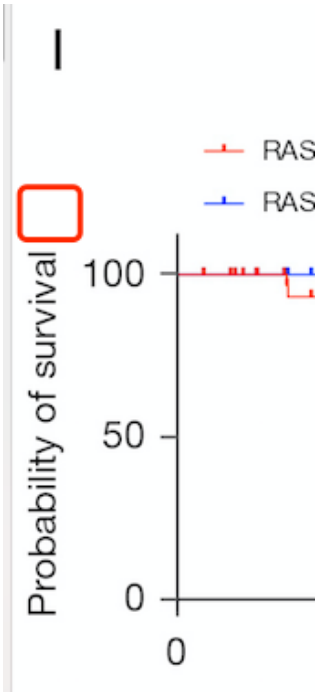
e. We suggest adding a line to indicate "****" clearly.



Reply: *Thanks, we have added the basket.*

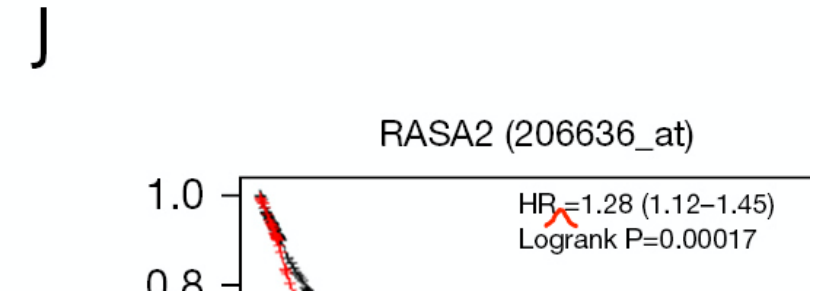
such as:

e. Please add the unit “%” for the Y-axis.



Reply: *Thanks, we have added it.*

f. Please add (95 % CI) after "HR".



Reply: *We cannot edit the original file, therefore we put 95%CI ahead of HR.*

g. Please confirm if this URL is necessary here.

556 with RASA2 high or low expression from January 2016 and June 2018. (J) KM plot of overall survival in
557 patients with different RASA2 expression levels. (https://kmplot.com/analysis/). Significance was
558 determined by Log-rank (Mantel-Cox) test (I) or t test (A and G) and is shown as *p < 0.05, **p < 0.01and

Reply: *We confirmed the URL is necessary.*

3. Figure 2

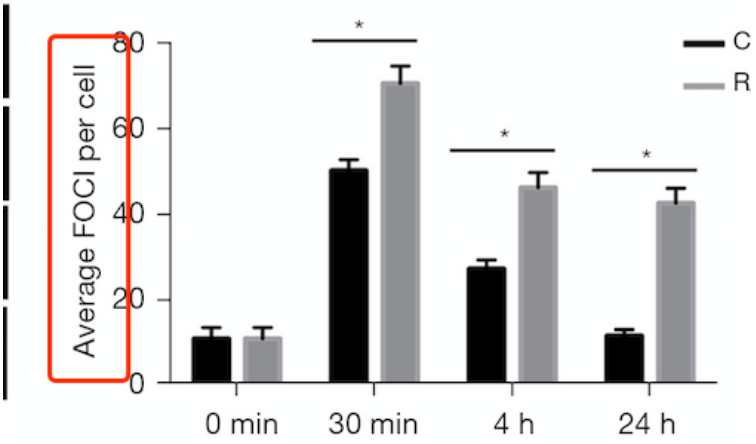
a. Please indicate the staining method of figure 2B in the figure legend.

Reply: *We have added the crystal violet (0.5% w/v) staining in the figure legend.*

b. Please spell out the FOCI in figure legend.

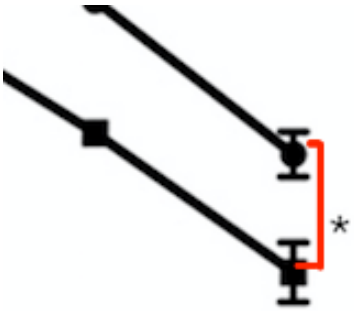
Reply: *We double checked, FOCI is not a abbreviation word. For better understanding, we have explained foci in the figure legend.*

c. Please add unit for the Y-axis in figure 2A.



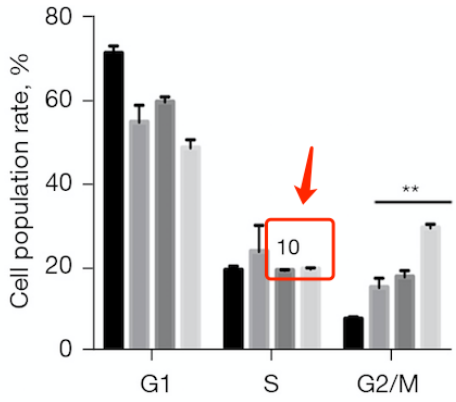
Reply: *We have added the unit..*

b. We suggest adding a line to indicate * clearly in figure 2B.



Reply: *We have added this line.*

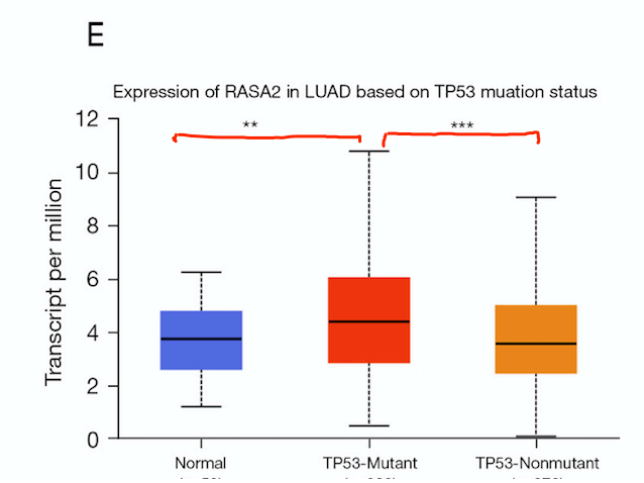
c. Please confirm whether this ‘10’ is correct in figure 2D.



Reply: *We have deleted this typing error.*

4. Figure 3

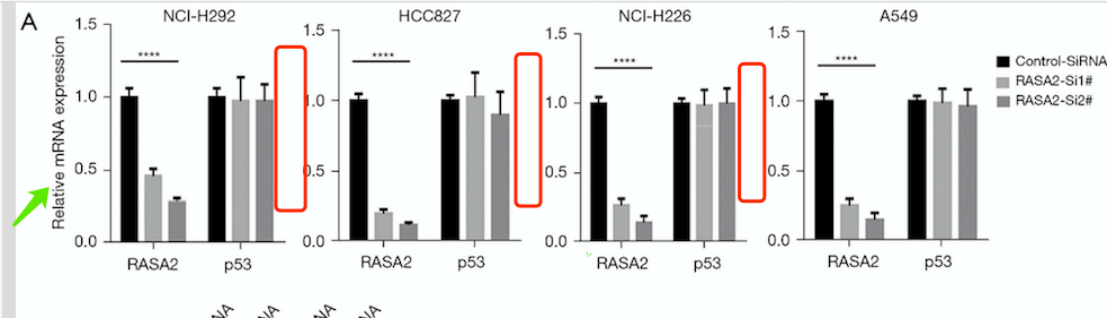
Please confirm if "**" means comparison between Normal and TP53-Mutant, and "***" means comparison between TP53-Mutant and TP53-Nonmuntant. If so, we suggest adding a line to indicate them clearly.



Reply: *We have added this basket.*

5. Figure 4

The description of Y-axis should also be added for the other three plots.

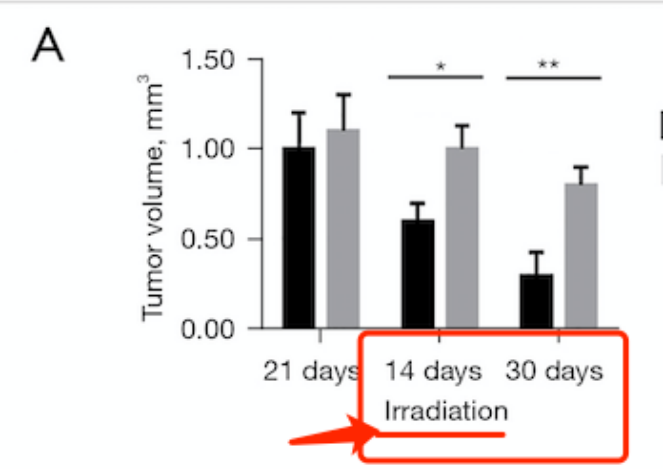


Reply: *We have added the Y-axis.*

6. Figure 6

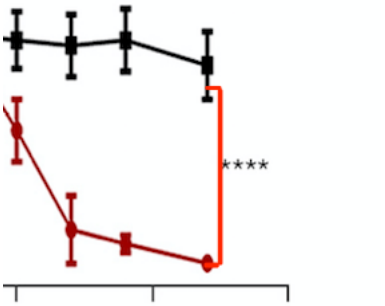
a. Please add “Injection” under “21 days” in figure 6A, or delete the “Irradiation”.

668 Figure 6 Knockdown of RASA2 enhanced radiosensitivity in *vivo*. (A) Left panel: BLI images of mice
669 bearing cancer cells (NCI-H226) with RASA2-control or -knockdown tumors. Mice (n=3) were imaged 21
670 days after injection and 14 and 30 days after irradiation. Irradiation: 24 Gy (1.6 Gy/day × 15 days). Right
671 panel: quantification of tumor volume. One of three representative experiments is shown. (B) IHC staining

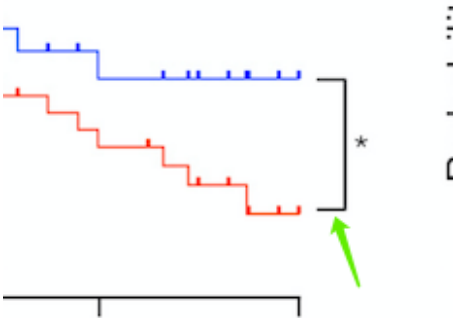


Reply: *We have added the information on the figure..*

b. We suggest adding a line to indicate "****" clearly in figure 6C.



Such as:



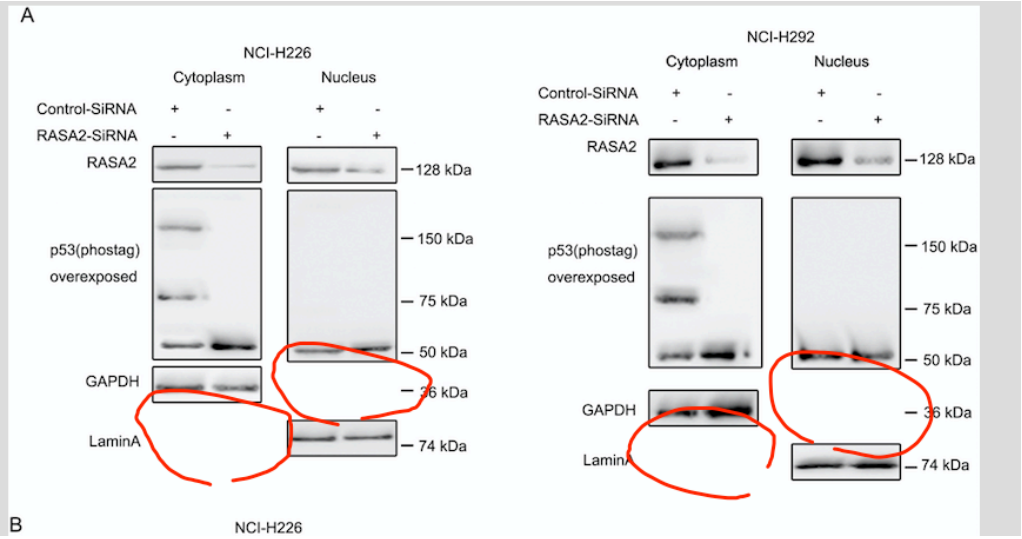
Reply: *We have added this line.*

7. Supplementary Figure 1C is not clear enough for publication. It would be much appreciated if you could provide it with a higher resolution as possible as you could. The preferred format is JPG or TIFF.

Reply: *Thank you so much for pointing this out, we try our best to provide the best quality of those plots.*

8. Figure S2

Please confirm if these two images are correct.



Reply: *We confirmed they were correct.*

9. Please reedit the layout of table S2. One content in one box.

719 Supplementary Table 2 Patient characteristics

Characteristic	Total N=205
Age (years), median (range)	62.3(27-86)
Sex	
Male	132
Female	73
Stage	
IIIa	123
IVa	82
Tumor location	
Left Lung	90
Right Lung	115
RECIST	
CR	44
PR	102
SD	37
PD	22

720 Click or tap here to enter text.

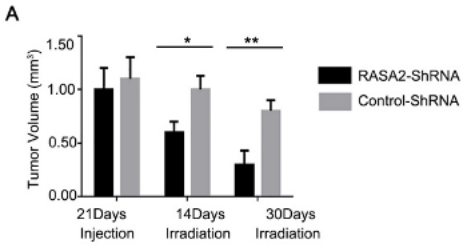
Reply: *We have edited it.*

10. Please explain all abbreviations used in table S1 and S2. e.g., RASA2, RECIST, SD, SE, CR, PR, SD, PD.

Reply: *We have explained it.*

11. Figure 6A

The legend of Figure 6A does not seem to match the Figure 6A. Please check.



832 Figure 6 Knockdown of RASA2 enhanced radiosensitivity in *vivo*. (A) Left panel: BLI images of mice
833 bearing cancer cells (NCI-H226) with RASA2-control or -knockdown tumors. Mice (n=3) were imaged 21
834 days after injection and 14 and 30 days after irradiation. Irradiation: 24 Gy (1.6 Gy/day × 15 days). Right
835 panel: quantification of tumor volume. One of three representative experiments is shown. (B) IHC staining
836 with HE for RASA2 in tumor sections (magnification, 400×). One of three representative experiments is

Reply: We are sorry for the confusion here and already corrected it.