Peer Review File

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Reviewer A

Comment 1: *Conclusion in abstract must be stake out. Conclusion is superficial, it does not show the main findings related to pathways more dysregulated in radioresistance HER2-positive breast cancer, ideally associated to biological properties of the cells.*

Reply 1:

We thank the reviewer for the valuable and thoughtful comments. We have re-written the abstract section in the revised manuscript according to the Reviewer's suggestions.

Changes in the text: We have changed the abstract section with the re-written one.

Comment 2: *In some reports there are evidences that in radioresistant cells the expression of HER2 or other membrane-receptors might be altered in response to radiotherapy. Authors must verify the expression of HER2 in radioresistant cells as a key mechanism to acquired radioresistnace.*

Reply 2:

Thank you for your suggestions. Indeed, the expression of HER2 or other membrane-receptors might be altered in response to radiotherapy. However, the literature confirmed the expression of HER2 was associated with radioresistance in breast cancer cells (1-4). Based on these studies, we chose the HER2-positive cancer cell line as the focus of our study. In this study, we only discovered the radioresistant phenotypes and possible mechanism of acquired radioresistance in breast cancer cells with HER2 overexpression. The key mechanism of acquired radioresistance will be investigated in future studies.

Comment 3: *Authors conclude that their method for establish radioresistant cell lines was "new" method ("with a new method") However, the strategy for established the radioresistant model isn't a new method, there are several reports in breast and other cancer models in which use the method described in this reports. In any case, authors should bediscussed which are the differences between properties of cells obtained in their model and others models in which only reach 40 Gy or less.*

Reply 3:

In previous studies, radioresistant breast cancer cells were established with fractionated irradiation (40 Gy, 20 fractions over 4 weeks) (5). In our study, the majority of cells did not survive after 40 Gy (20 fractions over 4 weeks) irradiation. The survived cells were then incubated for 4 weeks, and subsequently irradiated for further 4 weeks (40 Gy in 20 fractions). Considering our radioresistant model is similar to the repeated radiotherapy used clinically, we described our model as a new radioresistant model to distinguish from the previous methods.

Distinct differences in the acquired properties between our model and other models which received less than or equal to 40 Gy or less were observed. For example, Russell et al. established acquired radioresistant cells with fractionated dose (total 40 Gy) and observed that these cells reverted to wild-type after sub-cultured for 10-20 passages in the absence of

irradiation (5). In contrast, the radioresistant phenotypes of NF639R cells persisted after 30 passages in our study. It is not straightforward to discuss the differences between the properities of cells obtained in our model and other models, but we nonetheless compared the the properties of NF639R cells with those of NF639R$^{40\ Gy}$ cells. When compared with NF639R$^{40\ Gy}$ cells, NF639R cells displayed distinctly enhanced migration ability (Fig.5A, B in the revised manuscript) and lower basal level of ROS (Fig.5C, D in the revised manuscript). We have added these results in revised manucript.

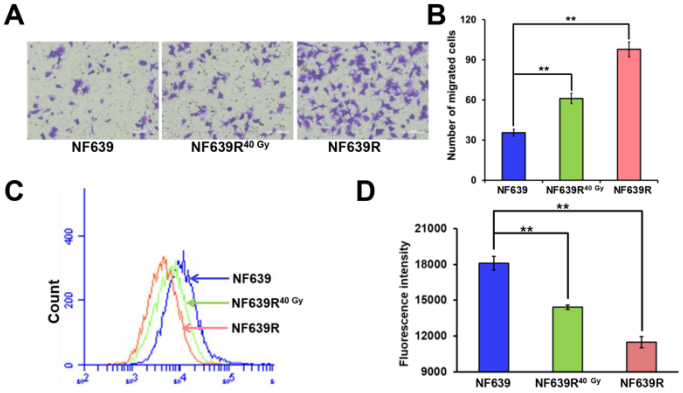


Figure 5 in the revised manuscript

Comparison of malignancy between NF639R$^{40\ Gy}$ cells and NF639R cells. A, Representative images of migrated NF639, NF639R$^{40\ Gy}$ and NF639R cells. B, Quantification of migrated NF639, NF639R$^{40\ Gy}$ and NF639R cells. C-D, Detection of ROS with CellROX deep red probe in NF639, NF639R$^{40\ Gy}$ and NF639R cells. C, Histogram profiles of NF639, NF639R$^{40\ Gy}$ and NF639R cells obtained using flow cytometry. D, Quantification of mean fluorescence intensity in NF639, NF639R$^{40\ Gy}$ and NF639R cells. All data are presented as the mean ± SD from three independent experiments. **: $p < 0.01$.

Changes in the text: We have added these changes in line 389 to line 395 in page 16 in the revised manuscript.

Comment 5: *Importantly, the expression of some genes dysregulated in cell line NF639R must be analyzed in the first schedule [after receiving first 40 Gy (20 fractions over 4 weeks)] in order to approaches the speculation: "We speculate that this strategy might be beneficial for prolonging the maintenance of acquired radioresistance, and this would have potential clinical implications since the restart of radiotherapy with short-term recurrence after radiotherapy may lead to increased radioresistance and promote tumor progress". These analysis may support the main idea to more aggressive phenotype of the cells to molecular level. Moreover, authors must be evaluated basal level of ROS in the first schedule [after receiving first 40 Gy (20 fractions over 4 weeks)] ROS levels is one of the principal mechanism of radioresistance. If the authors demonstrate that after the first schedule of radiotherapy the levels of ROS they might speculate about the better scheme of radiation therapies in breast cancer.*

Reply 5:

To confirm that two schedules of radiotherapy (40 Gy per schedule) rendered NF639 cells more aggressive, we evaluated the migration ability of NF639R cells and NF639R$^{40\ Gy}$ cells. The results showed that the migration ability of NF639R cells was distinctly enhanced compared with NF639R$^{40\ Gy}$ cells, suggesting that the subsequent irradiation (40 Gy) further promoted tumor progress. Furthermore, we also detected different espression of some genes associated with tumor progress, which included a postive prognostic indicator (HOXD13), two

inducers of radioresistance (ALDH3A1, and NQO1), one inducer of cell migration (TGFBI) and two suppressors of cell migration (SDPR and KLK10) with QqPCR in NF639R$^{40\ Gy}$ cells and NF639R cells (6-12). The results showed that the expression of ALDH3A1, TGFBI and NQO1 were significantly increased, but expression of SDPR, KLK10 and HOXD13 were decreased in both NF639R$^{40\ Gy}$ and NF639R cells. When compared with NF639R$^{40\ Gy}$ cells, higher expressions of ALDH3A1, TGFBI and NQO1 but lower expressions of SDPR and HOXD13 were observed in NF639R cells, and these results further supported our speculation. These results were added as Figures 5A, B, E, F in the revised manuscript.

As per the Reviewer's suggestion, we also evaluated the basal level of ROS in radioresistant cells after first or second 40 Gy irradiation. We detected lower basal level of ROS in NF639R cells than that in NF639R$^{40\ Gy}$ cells. We hope these results are helpful to designing radiotherapy schemes for treating breast cancers. These results have been added as Figure 5C, D in the revised manuscript.

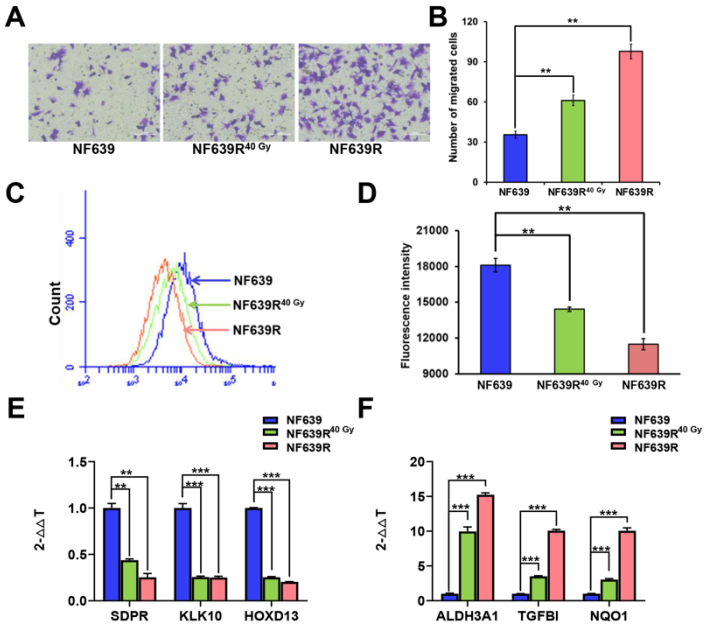


Figure 5 in the revised manuscript

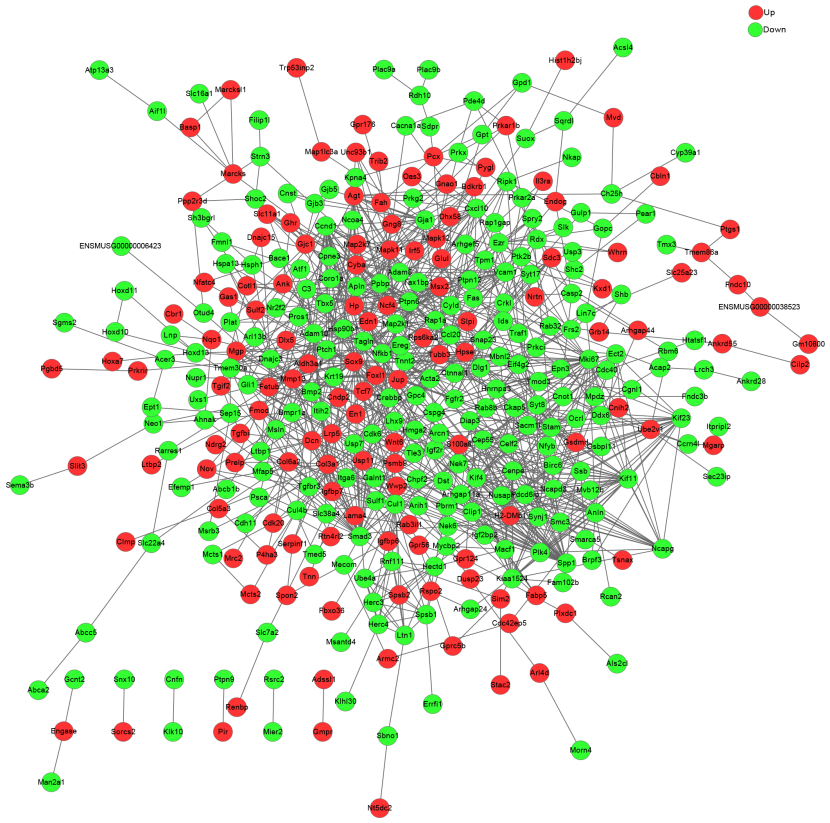
Comparison of malignancy between NF639R$^{40\ Gy}$ cells and NF639R cells. A, Representative images of migrated NF639, NF639R$^{40\ Gy}$ and NF639R cells. B, Quantification of migrated NF639, NF639R$^{40\ Gy}$ and NF639R cells. C-D, Detection of ROS with CellROX deep red probe in NF639, NF639R$^{40\ Gy}$ and NF639R cells. C, Histogram profiles of NF639, NF639R$^{40\ Gy}$ and NF639R cells obtained using flow cytometry. D, Quantification of mean fluorescence intensity in NF639, NF639R$^{40\ Gy}$ and NF639R cells. E-F, qPCR validation of some genes associated with tumor progress in NF639, NF639R$^{40\ Gy}$ and NF639R cells. E, Validation of upregulated genes including two genes (ALDH3A1 and NQO1) promoting radioresistance and one gene (TGFBI) promoting migration. F, Validation of downregulated genes including a prognostic indicator (HOXD13) and two genes (SDPR and KLK10) that inhibit migration. All data are presented as the mean ± SD from three independent experiments. **: $p < 0.01$, ***: $p < 0.001$.

Changes in the text: We have added these changes in line 389 to line 404 in page 17 in the revised manuscript.

Comment 6: Authors showed the transcriptome analysis, they should showed a network of protein interaction of the product of genes deregulated. MicroRNAs in cancer are the most enrichment KEGG pathway, however authors didn't discuss the finding.

Reply 6:

We thank for the Reviewer's suggestion. We have added a network of protein interaction of the product of genes deregulated in NF639R as supplementary Figure 1 in the revised manuscript. Indeed, microRNAs in cancers are the most enrichment KEGG pathway, and microRNAs maybe play an important role in the acquired radioresistance. As per the Reviewer's suggestion, we have discussed our results in the section of discussion in the revised manuscript. To indentify the key microRNAs, we will also need to analyze the effects of microRNAs on radioresistance by using microRNA-seq in future studies.



Supplementary Figure 1 in the revised manuscript

Protein-protein interaction network of the differentially expressed genes between NF639 and NF639R cells. Circles represent proteins, while lines represent strong association between proteins. Red color means upregulated while green color means downregulated.

Changes in the text: We have added these changes in line 336 to line 338 in page 14 in the revised manuscript.

Reviewer B

Comment 1: First of all, the title should report that the breast cancer model used is mouse.

Reply 1:

We thank the reviewer for the good suggestions. We have changed the title to "Properties and Gene Expression Profiling of Acquired Radioresistance in Mouse Breast Cancer Cells" in the revised manuscript.

Changes in the text: We have added these changes in line 2 in page 1 in the revised manuscript.

Comment 2: As regards colony formation assay (Fig. 1C), were the SF of the NF639 parental cell line, processed immediately after irradiation, compared with the SF of the NF639R cell line? Please, explain better in the text the comparison made between the survival curves at the level of time post irradiation analyzed.

Reply 2:

In our study, NF639 and MF639R cells were irradiated with 0, 2, 4, or 6 Gy of X-ray. After 10 days, SF of NF639 and NF639R cells were calculated. We then compared SF of NF639R and NF639 cells to evaluate the radioresistance. It is established that reproductive cell death is depicted as the main form of cell death induced by ionizing radiation (13). In the process of establishing the radioresistant model, the cells were weak after 40 or 80 Gy irradiation and many dead cells were observed. It was not practical to perform colony survival assay immediately after 40 or 80 Gy irradiation as the plating efficiency was too low. Thus, some recovery time was needed and the colony survival assay was then performed to allow comparisons between the SF of NF639 and NF639R cells. In our study, the recovery time was three months.

Changes in the text: We have added these changes in line 153 to line 160 in page 7 in the revised manuscript.

Comment 3: MN assay: which time point post irradiation for the study of MN on the two cell lines, NF639R and NF639 parental, was analyzed? This information should be added in the manuscript.

Reply 3:

We are very sorry for not expressing this clearly. MN assay was performed at three months after 80 Gy irradiation. At this timepoint, the irradiated cells recovered and resumed proliferation. We fixed the irradiated (0, 2 or 4 Gy) NF639 and NF639R cells at 48 h post irradiation and stained them to facilitate counting of micronuclei in binucleated cells. We have added this information in the revised manuscript.

Changes in the text: We have added these changes in line146to line 147 in page 6 in the revised manuscript.

Comment 4: Fig.2: the quality and magnitude of the cell micrographs should be improved in order to better show in detail the morphological differences observed between the two cell lines.

Reply 4:

We thank for the Reviewer's suggestion. We have replaced these images with better ones (Fig.2A in the revised manuscript) to clearly show the observed morphological differences between the two cell lines in the revised manuscript.

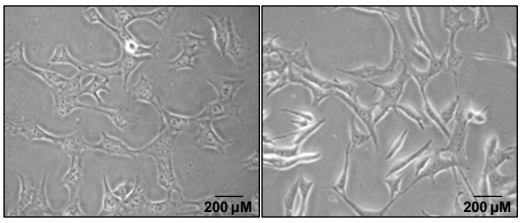


Figure 2A in the revised manuscript. Morphology of NF639 and NF639R cells.

Changes in the text: We have added these changes in line 286 to line 288 in page 12 in the revised manuscript.

Comment 5: Intracellular ROS detection: the authors should specify at what time post irradiation they determined the intracellular ROS concentration on both cell lines.

Reply 5:

We are very sorry for not having explained this clearly. We detected the intracellular ROS detection at three months after 80 Gy irradiation. Since we compared the basal level of ROS in NF639 and NF639R cells, the cells were not treated with any further radiation. We have added this information in the revised manuscript.

Changes in the text: We have added these changes in line 389 to line 395 in page 16 in the revised manuscript.

Comment 6: As regards qRT-PCR, the authors showed for the qPCR validation, nine upregulated and seven downregulated genes randomly selected from the top 100 differentially expressed genes. However, among the selected genes, with the exception of TGFb, those genes important to strengthen both the hypothesis of an acquired EMT and of CSC characteristics by the NF639R cells, are missing. Therefore, qRT-PCR experiments for some EMT and CSC biomarkers should be addressed.

Reply 6:

According to the Reviewer's suggestion, we detected the differentially expressed genes associated with EMT (Aldh3a1, Sox9, Mapk12, Wnt6 and TCF7) and CSC (Msx2, Gja1 and Gjb3) with QPCR. Previous studies have indicated that Aldh3a1, Sox9, Mapk12, Wnt6 and TCF7 could promote CSC phenotypes and Msx2 could promote EMT phenotypes (14-19). Gja1 and Gjb3 have also been reported to inhibit EMT phenotypes (20, 21). Our results showed increased expressions of Aldh3a1, Sox9, Mapk12, Wnt6, TCF7 and Msx2 but decreased expressions of Gja1 and Gjb3 in NF639R cells, which supported our hypothesis of acquired EMT and of CSC characteristics in the NF639R cells. These results were added into the revised manuscript as Figure 5I, J in the revised manuscript.

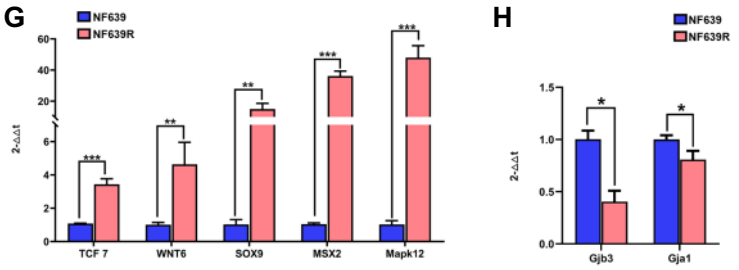


Figure 5(I-J) in the revised manuscript. qPCR validation of genes associated with EMT and CSC.
G, Validation of upregulated genes including four genes (Sox9、Mapk12、Wnt6 and TCF7) that promote CSC phenotypes and one gene (Msx2) that promote EMT phenotypes. H, Validation of downregulated genes including two genes (Gja1 and Gjb3) that inhibit EMT phenotypes. Data are expressed as mean ± SD, n = 3, each with triplicate samples. All data are presented as the mean ± SD from three independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Changes in the text: We have added these changes in line 395 to line 404 in page 17 in the revised manuscript.

Reviewer C

Comment 1: Overall, this is a descriptive study performed in a single cell line, which usually is considered not scientifically sound. Additional cell lines and/or mechanistic studies are necessary.

Reply 1:

We thank for the Reviwer's suggestion. In fact, a number of previous similar studies also employed only a single radioresistant cell line to investigate the possible molecular mechanisms of radioresisitance (22,23).In a number of other studies, although two or more radoresistant cell lines were established, the high-throughput measurements were only performed in one of the cell lines to explore the mechanisms with RNA sequencing or microarray (24, 25). Furthermore, the present work was only a preliminary study to establish a radioresistant tumor cell line and to explore potential signaling molecules, and we will further look for key signaling molecules from our preliminary results to explore the mechanism of radioresistance in the next study. We sincerely hope that the reviewer could agree and sympathize with our situation.

Changes in the text: We have added these changes in line 330 to line 333 in page 140 in the revised manuscript.

Comment 2: The rationale the authors give to use this cell line is that reportedly HER2 positive breast cancer is more radioresistant. However, of the 2 references given for this statement, one is a review (with wrong page numbers) which does not make any remarks on radioresistance, while the other finds that there are no statistically significant differences (p=0.21) between breast cancer subtypes. If HER2 positivity was an important rationale for this study, the authors should have compared HER2 positive and negative cell lines, possibly by knocking out HER2 in the same cell line.

Reply 2:

We are very sorry for the improper references. We have replaced these improper references (Reference 3, 4 in the original manuscript) with new ones (Reference 3,4 in the revised manuscript). In these previous studies, HER2 have been confirmed to play an important role in radioresistance of breast cancer. Based on these previous researches, we chose the HER2-positive cancer cell line in our study. In this study, we only discovered the radioresistant phenotypes and possible mechanism of acquired radioresistance in breast cancer cells with HER2 overexpression. Our experimental results could not prove that expression of HER2 in radioresistant cells was a key mechanism to acquired radioresistnace. Since the establishment of stable HER2 knockdown cell line and the induction of acquired radioresistance would need

a long time, we will investigate the role of HER2 in mediating subsequent acquired radioresistance in a future study. We sincerely hope that the reviewer could agree and sympathize with our situation.

Comment 3: No attempts are made to assess whether the changes found in radioresistant NF639 cells are cause of consequence, e.g. by inhibiting pathways or knockdown of relevant genes.

Reply 3:

We thank the reviewer for the valuable and thoughtful comments. In our study, activation of some signal pathways associated with anti-apoptosis, antioxidation, tumor stem cells and energy metabolism were observed in our established NF639R cells, and these pathways have also been reported to be involved in acquired radioresistance in previous studies (26-30). In our study, we only speculated that multiple pathways may be involved in the formation of acquired radioresistance. At present, we are also carrying out further validation of these pathways by knockdown of relevant genes, but it may take a long time. Therefore, we are really sorry that we cannot confirm the pathways playing the key role in the formation of radioresistance in this study. We will further explore the key mechanism of radioresistance in the next study. We sincerely hope that the reviewer could agree and sympathize with our situation.

Comment 4: 2GY induces approximately 30% cell death (figure 1C). After 20 fractions, even taking into account some recovery over the weekend, less than 0.2% of cells would have survived. How many cells did the authors start with? Was the identity of the cells after irradiation confirmed using STR analyses, given the strong selective pressure of the applied 80 GYs?

Reply 4:

We thank the reviewer for the valuable and thoughtful comments. Reproductive death is also depicted as the inportant form of cell death induced by ionizing radiation (31,32). As such, colony formation assay (Figure 1C in the revised manuscript), an *in vitro* cell survival assay based on the ability of single cells to grow into colonies, was regarded as the "golden" standard to determine cell reproductive death after irradiation (33). In colony formation experiment, 300 cells were seeded into 60 mm Petri dishes. The adherent cells were then irradiated with 0-6 Gy of X-ray. After 10 days, the number of visible colonies (more than 50 cells per colony) but not the cell number was counted. In fact, even though 300 cells were seeded into 60 mm Petri dishes, more than 300 cells were observed at 10 days after 6 Gy radiation. In our study, 4×10^6 cells were seeded into 10 cm dish before the fractionated radiation. In fact, our results showed that 8 Gy X-ray irradiation could induce 30% cell death (Fig. 2E in the revised manuscript). Therefore, a few cells could survive after a radiotherapy schedule (40 Gy in 20 fractions), and the survived cells proliferated again and could be used for the second radiotherapy schedule (40 Gy in 20 fractions).

The short tandem repeat (STR) profiling which relies on a PCR-based assay examining polymorphic tetranucleotide or pentanucleotide repeats was widely used for authentication of human cell lines. In our study, we did not perform the STR analysis. However, after receiving the strong selective pressure of the applied 80 Gy irradiation, expression of more than 50,000 genes were detected by mRNA-seq and only 490 genes (0.95%, 490/51826) exhibited significant differences between NF639 and NF639R. These results confirmed that the NF639R

cell line was derived from the NF639 cell line.
Changes in the text: We have added these changes in line 153 to line 157 in page 7 in the revised manuscript.