Author's response to reviews

Title: Inhibition of Radiation Induced Migration of Human Head and Neck Squamous Cell Carcinoma by Blocking EGF Receptor Pathways

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Author's response to reviews: see over
Dear Sabina Alam,

please find included the revised version of our manuscript. The text that has been modified according to questions and recommendations of the reviewers is highlighted in yellow. Also, we have done another experiment with a modified Boyden chamber. Please also find attached the point by point response to the individual remarks.

On behalf of the authors

A. Pickhard
Reviewer: Deborah Citrin

Major compulsory revisions:

1) General: There are a number of grammatical mistakes throughout the manuscript and figures. These should be corrected.

*We have checked the manuscript and an English native speaker had revised it.*

2) Tables and Figures: The manuscript describes references tables which are not present in the provided materials. These should be added.

*Reference tables are added*

3) Tables and Figures: Induction of these pathways after radiation, and inhibition of the targets of each agent is critical to the rationale of this study and the results the authors report.

The Western blot results should be presented in figure form.

*The Western blot results were presented in figure form (Figure 7)*

The description provided is complicated and difficult to understand.

*The description of the figures and tables was revised.*

4) Tables and Figures: The authors report that they do not observe phosphorylation of EGFR in their western blots. This is critical to the rational of the study in that they suggest inhibition of EGR alters migration. The 24 hour time point may not is the most appropriate time at which to assay this receptor and alternative time points should be considered or an alternative explanation of these results should be provided.
In our primary Western Blot experiments without FCS an activation of the EGFR was not detectable. When doing the western blots again with FCS added, an activation of the EGFR was observed (see Figure 7).

**Minor Essential Revisions:**

1) Background: The authors reference invasion and migration. They reference Camphausen et al in regards to Lewis Lung Model. This reference showed that a molecule liberated at the time of radiation affected dormant metastatic cells in the lung, not that radiation induced new metastases proving an abscopal effect. Although this is an excellent reference, this does not relate to migration or invasion.

   *This reference was removed.*

2) Methods: The energy and dose rate of the irradiation used should be reported.

   *Irradiation was performed at the Department of Radiotherapy (Technical University of Munich). Cells were X-irradiated with single doses of 2, 5 or 8 Gy with a Philips RT 100 (Philips, Amsterdam) operated at 300 kV with 1.4 mm copper half-value layer at a dose rate of approximately 1 Gy/min. The dose inhomogeneity was ±2%. The sham-treated group (0 Gy, control) was subjected to the same protocol as exposed cells (see page 5).*

3) Results: The authors state in the results that “The increase in migration by radiation was significantly reduced through stimulation with EGF as well as through inhibition with AG1478”. This is not what is evident from review of the figure and figure legend and does not make sense based on the hypothesis. Looking at the figures it appears that there is no effect or a small increase in migration after stimulation with EGF. This should be clarified in the text.

   *After stimulation with EGF, radiation does not seem to increase migration, in contrast to what happens with control cells. The same holds for inhibition through AG1478. The effect of radiation on migration thus seems to be less pronounced after stimulation with EGF or inhibition with AG1478 than in control cells. However, this*
reviewer is right that stimulation with EGF increases migration for 0 Gy. This is not contradictory to our statement, and we also mention this increase in the manuscript. We reformulated the corresponding paragraph to clarify this point (see page 9).

4) Results: The authors state that the use of all 3 inhibitors of downstream pathways blocked migration in all three cell lines. Looking at figure 4, it appears that treatment of the BHY cell line with PD98059 increased migration without and with radiation. This should be described in the results and the possible rationale for this effect should be discussed in the discussion.

We mean here that, after inhibition, migration does not increase with increasing radiation dose. In other words, while migration significantly increases with radiation dose in controls, this is not observed after treatment with the 3 inhibitors. The term “blocked” in our first version was probably inappropriate and confusing. We rephrased the corresponding paragraph as well as the legend of Table 1 to clarify this important point (see page 10).

5) Discussion: The rationale for evaluating proliferation is not well described and the implications of these results are not discussed. It is clear that proliferation would be an important variable to evaluate in terms of the scratch test as increased proliferation could result in filling in of edges and an increase in migration in setting of decreased proliferation is a more powerful result. This should be discussed.

We discussed this point (see page 12).

Discretionary Revisions:
1) In the results section the authors report the decrease in MAPK signaling after the inhibition of PI3K. It would be appropriate to discuss this in terms of possible off-target effects.

We discussed this effect in the discussion section (see page 13).
Reviewer: Maria Sundvall

Major compulsory revisions:

1) The authors discuss and make conclusions based on western blot data not shown. Please include these data as a new figure in the manuscript.

*The Western blot results were presented in figure form (Figure 7).*

2) The data presented in table 1 is not clear. What is p-value 0.000.

*We are sorry, P-value <0.01 was meant and we revised it (see Table 1).*

Data of another time point can be omitted for simplicity.

*We now present the data for 12 hours only.*

3) Table 2 is not very informative. Instead of presenting absorbance values of one experiment, data should be processed further and presented e.g. as bars (like migration experiments in fig 2-4). The average value of at least triplicate wells of a representative experiment with SDs should be shown.

Is fig 5 already made of these absorbance values of CAL cell line in table 2?

Combine fig 5A and B to allow comparison between the data of irradiated and non-irradiated cells with or without inhibitor treatment. If all cell lines behave relatively similarly in MTT assay with or without inhibitors, **I think it is enough to show only data of the one representative cell line as fig 5 and delete table 2.** Since inhibitors were added 12 h prior to radiation treatment, include also time point 0 (measurement just before radiation).

*We followed this request and deleted Table 2.*

4) The authors say that activation of EGFR is important for the migration of HNSCC cells after irradiation. However, they did not see any activation of EGFR by
irradiation. Moreover, they show that stimulation of cells with EGFR ligand EGF inhibits rather than stimulates migration induced by irradiation. This seems inconsistent. The authors should give a putative explanation for this or provide additional data.

In our primary Western Blot experiments without FCS an activation of the EGFR was not detectable. When doing the western blots again with FCS added, an activation of the EGFR was observed (see Figure 7).

What we mean here is that, after stimulation with EGF, radiation does not seem to increase migration, in contrast to what happens with control cells. The same holds for inhibition through AG1478. The effect of radiation on migration thus seems to be less pronounced after stimulation with EGF or inhibition with AG1478 than in control cells. However, this reviewer is right that stimulation with EGF increases migration for 0 Gy. This is not contradictory to our statement, and we also mention this increase in the manuscript. We reformulated the corresponding paragraph to clarify this point (see page 9).

5) It is not clear how the migrating cells were counted. Representative photographic images of the wound healing assay -/+ irradiation should be shown (e.g. with one cell line)

The migrating cells/photographic images of the wound healing assay -/+ irradiation were presented in figure form (Figure 2).

6) Given the importance and novelty of the finding that irradiation stimulates migration of HNSCC cells the basic finding should be confirmed with another type of assay, e.g. with boyden chamber migration assay, or alternatively the authors should analyze whether irradiation also promotes invasion of theses cells in vitro e.g. through matrigel.

We have done another experiment with a modified Boyden chamber. As we had to establish the test in our lab, we did the experiment only for the radiation induced migration. These results confirm the data of the scratch test (see page 9).
7) There is inconsistency between methods, figures and figure text. For example, in methods section, 1st chapter, the authors state that all measurements were made in nine experiments and after culturing cells for 24 h after irradiation. To what assay is this referring? According to methods, migration assay was analyzed at 12 and 24 h time points after IR, and MTT assay measurements were made in eight experiments and incubated 12, 24 or 72 h after IR. And table 2 and figure 5 show 24, 48 and 72 h time points for MTT assay? These need to be clarified.

*In methods section, 1st chapter, the sentence ‘After irradiation, cells were cultured for another 24 hours. All measurements were made in nine experiments’ was irritating, so that we comment the further treatment of the cells by the separate chapter (MTT and wound healing assay).*

*The different time points were omitted by the wound healing assay, as described in point 2. The MTT test was made at 12, 24 and 72 hrs. The legends of Table 2 and Figure 5 were wrong. We corrected them (see Figure 6).*

8) The results and discussion section about western blot data should be rewritten to make it more coherent. Please check also these in Results section: i) What does the word “probe” mean in the title? Cells?; ii) Why did PD98059 associate with a decrease of phosphor-MAPK at 0 time point? When was the inhibitor added?, iii) The last sentence “in all probes…” is unclear.

*The Western blot results were presented in figure form (Figure 7). Also, the description was revised (see page 11).*

9) Figure 1 should be improved. For example, MAPK=Erk and PD98059 inhibits MEK1 (= MAP kinase kinase). This error needs to be corrected. The classical components of MAPK cascade should be illustrated (Ras-Raf-MEK-Erk). Indicate also cell surface to the picture. What are the black circles attached to EGFR, Raf, MAPK and PKB?

*Figure 1 was revised.*
10) More up-to-date discussion about the role of EGFR inhibitors in the treatment of HNSCC is needed in Background and Discussion sections. For example, the anti-EGFR antibody cetuximab has already been approved by the FDA and EMEA for treatment of locally advanced HNSCC with concomitant radiotherapy. Include data and reference of the landmark phase III trial by Bonner et al. demonstrating survival benefit of concomitant use of cetuximab with radiation compared to radiation alone in treatment of patients with advanced HNSCC in the manuscript. Discuss the current clinical status of EGFR targeting antibodies and tyrosine kinase inhibitors as well as inhibitors of EGFR downstream signaling pathways components in combination with radiotherapy for treatment of HNSCC.

*We have updated the references.*

11) Please update the references. For example, reference 17 (published 2001) is rather old to cover recent molecular strategies.

*We have updated the references.*

**Minor Essential Revisions**

1) The same terms should be used throughout the text. For example, i) when both PKB/Akt and PI3K/Akt are used it is not clear that PKB is an alternative name for Akt whereas PI3K and Akt are different components of a signaling pathway; ii) the use of alternative names for the same molecule (e.g. phospho-Erk vs. phospho–MAPK) is misleading.

*The same terms are now used.*

2) Abstract – Results: It is misleading not to mention the results of EGF or inhibitors on irradiation induced migration.

*We are sorry, but due to the word count limit, it was impossible to explain all reactions of inhibitors in the abstract.*
3) Abstract – Conclusions: Specify “these” proteins in the last sentence.

*It was changed as suggested.*

4) In Background section, the 1\textsuperscript{st} paragraph: “Curative and adjuvant ..” Sentence is unclear and should be rewritten. Describe the anti-neoplastic properties of radiation as a separate sentence.

*We reformulated the sentence.*

5) In Background section, the 3\textsuperscript{rd} paragraph: “Also, Camphausen et al …” sentence is unclear and should be rewritten.

*This reference was removed.*

6) In Background section, the 5\textsuperscript{th} paragraph: In addition to reference 15 and 16, other references are needed to cover the studies that correlate molecular markers with radiation response. Please add also what is known of EGFR and its downstream signaling molecules as predictive markers for radiation response in HNSCC.

*We have updated the references.*

7) Background section, the 6\textsuperscript{th} paragraph: The sentence “Mutation in the cellular …” is unclear and should be rewritten.

*The sentence was removed.*

8) In figure 5 “kontrolle” → control

*It was corrected.*

9) Proteinkinase → protein kinase

*It was corrected.*
10) In Discussion section, the 3rd paragraph: “blockade of Akt by rapamycin” is not correct.

*It was corrected.*

11) In Discussion section, the 5th paragraph: “stadium” → stage

*It was corrected.*