Author's response to reviews

Title: Tumor Cells With Low Proteasome Subunit Expression Predict Overall Survival in Head and Neck Cancer Patients

Authors:

Chann Lagadec (clagadec@gmail.com)
Erina Vlashi (evlashi@mednet.ucla.edu)
Sunita Bhuta (sbhuta@mednet.ucla.edu)
Chi Lai (chilai@mednet.ucla.edu)
Paul Mischel (pmischel@ucsd.edu)
Martin Werner (martin.werner@uniklinik-freiburg.de)
Michael Henke (henke@uni-freiburg.de)
Frank Pajonk (fpajonk@mednet.ucla.edu)

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Author's response to reviews: see over
Dear Sir/Madame,

We are grateful for the opportunity to resubmit our manuscript (1517383364107469) entitled, “Tumor Cells With Low Proteasome Subunit Expression and Overall Survival in Head and Neck Cancer Patients” for reconsideration in BMC Cancer. We appreciate the thoughtful comments the reviewers have made and have included their suggestions in the revised version of the manuscript. We feel their comments have significantly improved our manuscript.

Below we will address the reviewers’ comments point-by-point, and hope that our manuscript is now suitable for publication.

Reviewer: Claire Rodriguez-Lafrasse

Major Compulsory Revisions:

Three major points require clarification:

1. Table 2 (clinical information) is missing

We apologize for the missing tables 2 and 3. They have been added to the manuscript.

2. In their previous work (Lagadec et al., Breast Canc Res, 2010), the authors compared the expression of low proteasome function (ZsGreen-cODC+ cells) with classical markers of CSC (CD24-/low/CD44high) in a “cancer initiating cell” subpopulation of breast cancer cells and concluded that “not all CD24 /low/CD44high cells were positive for ZsGreen-cODC, indicating that ZsGreen-cODC+ cells constituted a sub-population of the CD24-/low/CD44high population”. In HNSCC, it is also fundamental to compare the expression of low proteasome function with that of the classically admitted markers of CSC (side population (Sun et al., 2010), CD44high (Facompre, 2012), ALDHhigh (Chen et al., 2010)...). Do the authors consider that ZsGreen-cODC+ cells are a part of, different from, or the totality of the cancer stem cell sub-population?

We have attempted to use CD44 as a cancer stem cell marker for HNSCC in the past. However, on paraffin-embedded patient specimen we found CD44 ubiquitously expressed on cancer cells at very high levels, suggesting that CD44 is not a marker that can enrich for CSCs in HNSCC patient samples (see the newly added figure 1d). CD44 was originally described by Prince and coworkers but is no longer used by this group.
The side population assay has the potential of Hoechst dye induced toxicity for the live cells. As a consequence, cells that do not exclude the dye may have lower tumorigenicity due to the toxicity of the dye but not as a consequence of their phenotype. Therefore, we do not use the SP assays to screen for CSCs.

The Aldefluor Assay cannot easily be performed with our reporter system since our ZsGreen-cODC reporter system and the Aldeflour assay’s substrate are based on green fluorescence. We attempted using DsRed-cODC instead of ZsGreen-cODC in HNSCC instead, but found overexpression of DsRed to be toxic to HNSCC. Furthermore, reported percentages of ALDH1+ cells in HNSCC established cell lines in the literature by the Prince group are highly variable between 0.5 and 14% and are unlikely to reflect the rare nature of CSCs in HNSCCs (Otolaryngol Head Neck Surg. 2013 Aug;149(2):252-60) if used alone.

As an alternative in response to this reviewer’s request we have used antibodies against ALDH1 and flow cytometry to assess overlap between cells with low proteasome activity (ZsGreen-cODC-pos) and ALDH1 expression. Data for SCC-6 and SCC-12 are very similar showing that ZsGreen-cODC+ cells are a subpopulation of the ALDH1+ cells (Figure 1e&f).

Overall, we believe that all assays that enrich for CSCs as defined by operational means have their role in CSC research and do overlap. Our system adds an easy tool to the field that can be conveniently utilized without the need for additional staining or manipulation of the cells and allows for in situ studying of these cells.

3. One strength of this paper is to confirm the results obtained in cell lines on biopsies of HNSCC patients. In the latter, they establish Kaplan-Meier curves for survival and locoregional tumor control according to three levels of PMSD1 expression. Although the response of the different cell lines to fractionated irradiation was studied in terms of ZsGreen-cODC+ and tumor sphere formation, a link is missing between the low proteasome expression and radioresistance. Clonogenic cell survival performed on the low and high ZsGreen-cODC sub-population of the 6 cell lines should demonstrate if a relationship exists between low proteasome function and radioresistance.

While these experiments would indeed be useful in determining the relative radioresistance of ZsGreen-pos population versus ZsGreen-neg, these experiments are technically difficult and nearly impossible to perform. The reasons are:

- ZsGreen-cODC+ cells are very rare and require extremely long sorting times for a full dose curve, which reduce viability, and thus clonogenicity.
- Clonogenic assays for ZsG+ and ZsG- have to be performed under different culture conditions (monolayer vs sphere-forming capacity) to
maintain the phenotype of ZsG+ cells. While these experiments are useful to study e.g. the effect of hypoxia on the radiation response of both populations, we don’t think that survival curves from clonogenic assays and sphere-forming assays can be compared with each other directly.

Minor essential Revisions:

- The abstract is not sufficiently clear about the objectives of the paper

We modified the abstract to clearly state the objective of the study

- Fig 2b and 2c: maintain the same iconography for SCC12 and SCC6

We changed figure 2b accordingly

- Since all the experiments cannot been performed on all the cell lines (example fig2B,C,D), the choice of SCC6 and SCC12 as reference cell lines requires justification.

SCC6 and SCC12 have been chosen because the number of ZsGreen+ in monolayers in these two lines best reflected the range observed in the whole panel (with the exception of CAL33).

- The experimental protocol which enables fluorescence in cells grown as tumorspheres to be quantified is not detailed in the material & methods section.

We expanded the M&M section to explain the principle of the reporter system

- Error in typography of Fig 5e (there are 2 Fig 5d panels)

We have corrected Figure 2

- If a comparison is made between the number of cells in the windows of fluorescence measurement of the FACS profiles (fig 2e) and the mean results presented in fig 2b for ZsGreen-cODC+ and ZsGreen-cODC- populations, the results appear very different for SCC17b and at a lower level for SCC6 and FaDu. Could the authors justify it?

Figure 2B shows the mean results of experiments performed on sphere cultures whereas figure 2E shows FACS data from monolayer cultures. The latter was not stated in the figure legend and we have added this information.
- The experimental protocol in the legend for fig 2c,d is not clear. If I understand well from the text, cells were seeded at a clonal density for the formation of tumor spheres, 72 hours after 5 x 3 Gy irradiation of monolayers or tumorsphere cultures. We could have expected an increase in the population forming spheres from the non-irradiated (0 Gy) tumorsphere compared with cells grown as monolayers and thus containing fewer tumorsphere-initiating cells. This point requires discussion.

We actually observe this quite frequently in primary sphere cultures. Increased and sustained self-renewal is often more obvious in secondary and tertiary sphere cultures. However, this experiment solely demonstrates enrichment for sphere forming cells after fractionated RT.

Results in fig 2b are surprising (high variation in the % of ZsGreen-cODC+ cells in non-irradiated cells). Yet this experiment is essential to demonstrate the relationship between low proteasome expression, radioresistance and tumorsphere formation. It is difficult to conclude from the conflicting results in the two cell lines and the analysis of a third line should enable a conclusion to be reached.

The results are not surprising in the context of the radiation literature on both lines. SCC6 is known to be very radiosensitive (SF2Gy ~.3) while SCC12 are highly radioresistant (SF2Gy ~.8). Consequently, radiation does not enrich for CSCs if cells are subjected to fractionated RT. We have expanded the results section to highlight this point and added references to manuscripts showing the sensitivity of both lines.

- The clinical results clearly demonstrate a statistically significant relationship between PMSD1 expression and survival but are not significant when PMSD1 expression is compared with locoregional recurrence. The text in the result and discussion section should be modified so as not to give these two criteria an equivalent value.

We have revised the Discussion section accordingly. The Results section already clearly distinguished between local control and overall survival.

Reviewer: Douglas R. Spitz

Specific Comments:
1) The authors don't clearly explain the biochemical/biological argument behind why low proteosome expressing cells may be representative of cancer stem cells and render them resistant to radiation, relative to the rest of the cancer cells in the population that have high proteosome activity. Is it because they have low metabolic levels of reactive species that damage proteins so they turn proteins over more slowly? Some comment on this should be added.

We have added a paragraph to the Discussion section to comment on this observation.

2) In the section on x-irradiation what was the potential applied to the x-ray tube and the filtration used to generate the beam.

We have added this information to the M&M section.

3) How were spheres counted in each well when they were not attached?

The spheres were counted visually using an inverted microscope.

4) When cells were injected into animals did they form metastasis?

No. We did not observe any macroscopic metastasis during necropsy.

5) In the patients were follow analysis by FDG-PET imaging accomplished? Was there any correlation between SUV by PET imaging and CSCs or relapse?

The patients did not receive PET imaging. Since the study has a 9-year follow-up PET was not standard-of-care in these trials

6) Does the accumulation of the fluorescent fusion protein in cells cause toxicity?

No. We have not observed any toxicity related to ZsGreen. ZsGreen+ with very high expression of the fusion protein are perfectly viable.

7) Table 1 contains rather small numbers of animals/group. Which values are statistically different from other values on this table?
We have calculated the frequency of CSCs in both groups and found that the ZsGreen+ population contains significantly more CSCs. This was added to the manuscript.

8) It seems like the data with the patients that underwent successful resection is different than the ones without resection? Could the authors speculate why this is true?

We added a paragraph to the discussion section addressing this point.

9) The references many times are not following a standard format and some are missing volume numbers or have unnecessary information added to the reference. This is true for references 6, 7, 9, 27, 34 and maybe others. This needs to be checked carefully and corrected.

We have checked and corrected the references.

10) Figure 3 b is an awkward presentation. Why aren't the low, intermediate, and high proteosome expressors with N values clearly labeled on the figure? This needs to be labeled more clearly.

Figure 3b, c, and d have been edited accordingly.