Author's response to reviews

Title: Functional p53 is required for rapid restoration of daunorubicin-induced lesions of the spleen

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Version: 3 Date: 3 May 2013

Author's response to reviews: see over
Dear editor

Thank you for a thorough revision report on our manuscript. We have made several corrections based on the reviewer’s report, and have also restructured the abstract to comply with the BMC cancer format. We have also revised and edited the language, and hope that it is satisfactory. We have chosen to use UK-English. If this is not acceptable, we can change into US-English. Due to the extra experiments needed, we had to include L Myhren in the author list (she made spleen extracts and performed the immunoblotting). We hope this does not cause any problems.

Below are the reviewer’s comments, and our response to them (in red).

In general, we have changed our manuscript according to reviewer 1’s comments, which we found very useful. We believe that this has improved the manuscript. Reviewer 2 suggested two experiments to complement the data already present. We have examined death signalling downstream of p53 by western blotting from frozen spleen samples from the initial experiments. We have investigated the levels of p21 during the DNR treatment of the mice. We have also investigated the death promoters Bax, p63 and p73, the two latter often associated with p53. These data are presented in a new Fig. 3, and in a new paragraph at the end of the Results and Discussion section. Unfortunately, the wt-control mouse sample was not possible to use, but we believe that the immunoblots are still of significant interest, and merit to be presented.

The second experiment suggested (isolate splenocytes and silence p53 with siRNA) is not possible for us to perform on a short notice for several reasons: First, we have to breed animals for the experiment, which can take up to several months. Secondly, based on our extensive experience in transfecting leukaemia cell lines, we know that this can be very difficult depending on the cell line. To perform this on isolated splenocytes, that already have been removed from their natural environment, needs optimization that can take several months, and still not be successful. Also, there is a concern with silencing protein translation with siRNA as there still can be some protein left that can respond to the drug treatment. We hope that the data presented, where p53 is knocked out completely, is sufficient to convince the readers of the role of p53 in anthracycline-induced cell death in non-cancer tissues. We hope that the revised version is worthy publication in BMC cancer.

Below is our response to the reviewer’s comments (in red). Changes in the revised manuscript are also in red.

Sincerely,
On behalf of all authors

Lars Herfindal, PhD
Reviewer's report
Title:
Functional p53 is required for rapid restoration of daunorubicin-induced lesions of the spleen
Version: 1
Date: 25 March 2013

Reviewer:
Margareta Wilhelm
Reviewer's report:
In this manuscript Herfindal and colleagues describe that p53 is required for rapid restoration of daunorubicin-induced lesions in the spleen. The authors have treated p53 knockout and wt mice with daunorubicin and compared the onset of apoptosis and recovery after treatment. There are some concerns the authors have to respond to before publication, see comments below;

General comments:
- The manuscript need to be more carefully edited, in several places it is not clear whether they have used one or more mice in their experiments, often refers to mouse instead of mice. Also trp53 vs p53 nomenclature is not consistent.
- Thank you for pointing this out. We have gone through the manuscript and replaced errors and bad wordings. We have also revised the use of Trp53 and p53 so that Trp53 is used to name the gene, whereas p53 refers to the protein.
- The statistical data analysis need to be clarified ? In the bar diagrams there are stars which may be indicating significant values; however the p value cut off point (0.05, 0.01 etc) was not mentioned and no reference has been made to any significant values in the text.
- A short paragraph on statistic analysis has been included in the methods section, and the asterisks have been explained in the figure legends.

Specific comments:
Figure 1
-(A) The first sentence has to be changed, authors refer to (A) and (B) being wt and ko mice but this is not as it is in the figure.
-This has been corrected
1. The amount of apoptosis overall is relatively low, would like to see how much total cells death occurs, including necrosis.
- The cells were gated for AnnexinV and propidium iodide positive cells. Necrotic cells stain for both dyes, and are thus counted here. This has been clarified in the methods section.
2. It is not clear whether the cells were sorted for spleen-derived leukocytes before being treated or was the whole heterogeneous population used to investigate apoptosis. If not sorted then one should perhaps not refer to the population as spleen-derived leukocyte but rather splenocytes.
- It is debated which term is the most correct to use. Based on the arguments above, we agree with the reviewer that splenocytes is the correct term on non-sorted spleen-derived cells, and we have replaced spleen-derived leukocytes with splenocytes.
3. Spontaneous cell death can occur during the mechanical dissociation of primary tissue, a control or vehicle only sample should be included.

- The reviewer correctly points out that there will be some spontaneous cell death during the preparation of the samples. This has been recorded and subtracted from the data shown in Fig. 1. The spontaneous death in control samples were always less than 15%, and this information has now been included in the methods section.

4. Not clear why IDA was used, is it a positive control? Clarify in text or in figure caption. Also IDA concentrations and source is not mentioned in materials and method section.

- We have clarified this in the result and discussion section: Both drugs have been used in leukaemia therapy, but we continued to use DNR since this is the preferred drug in AML.

The concentration of IDA is mentioned in the figure legend. The supplier has been added in the Methods section.

(B,C)

1. A small figure legend would make it easier to grasp this results better

- We feel that the figure legend presents the necessary information to be able to understand the data in Figure 1B,C, but have shortened it some to increase readability.

2. The right plot representing spleen weight after ionizing radiation is not explained in material and methods at all (time of exposure, strength of radiation etc.). Its relevance is not clear, how is it linked to the findings and contribution of this paper. Moreover, the number of mice used for this experiment (n=2) is too few to make any conclusions.

- The right plots represent the spleen weight of animals 14 days after chemotherapy. This is now explained in the figure caption. Moreover, we have clarified this part of the figure in the main text (page 5). We have tried to avoid too much emphasis on these results, but feel that they could be of interest to the readers.

3. Why were the animals anesthetized rather than euthanized when the spleen was removed? And if anesthetized, what form of anesthesia was used?

- The mice were euthanized. This has been corrected in the Methods section.

Figure 2

For orientation purpose low magnification images of the spleen sections would be good. How were the pyknotic nuclei quantified? Were they counted in a specific area or within specific dimensions of the section? Do the stars in the bar diagrams refer to significant values? Then what are the p values and what is the p value cut off point (0.05?)?

- In order to be able to fit the figure into one page, we had to omit low magnification images of the spleen. Also, the mouse spleen is rather evenly organized into red and white pulp, and we felt that the images depicts representable areas. We hope that the figure gives sufficient information without low magnification images.

- We have added information on quantification of the pyknotic nuclei: The number of pyknotic nuclei in all the white pulp areas was counted and then divided by the number of white pulp regions. Lipofuscin-like pigmentation was quantified in the red pulp.
We have explained the asterisks in the diagrams. Thank you for pointing out this.

(A)
1. What was observed in animals treated for more or less than 3 days? What is the significance of using 3 day, does it have clinical relevance, if so need to be mentioned.
   - A typical therapy regime of DNR in AML patients consists of e.g. a total of three one-hour infusions during three to six days (Mayer et al., N Engl J Med, 1994 and several later publications). We wanted to study the damage in the spleen just after DNR therapy and the following days to find the recovery time and how p53 is involved in the damage and recovery of the spleen. This has now been explained further in the Results and Discussion section.

2. What was observed in the red pulp sections with these stains, why were the lymph nodules (white pulp) investigate here?
   - Pyknosis was mainly observed in the white pulp. We interpret that the increased lipofuscin-like pigments were signs of cell death in the red pulp. This has been clarified in the Results and Discussion section.

(B)
1. Is this white or red pulp section?
   - There was severe degeneration in both red and white pulp, and we have clarified this in the main text. In order to identify this properly, we have added arrows to identify the white and red pulp in the low-magnification images.

2. N=1? Were there more of these observations of this kind, if so need to be mentioned otherwise result lose significance.
   - These were observations from 3 wt and 4 null mice, but the images show representative sections. Since these were not quantified, we did not mention the number of mice, but this is now in the Results and Discussion section.

3. The two arrows are pointing to two different things.
   - The two arrowheads point to pyknotic nuclei, but those to the right were partly occluded by the arrow. We have moved this arrow to make it easier to see.

4. I recommend this figure either is clarified and improved on.
   - We have added more information to the figure, such as WP and RP to identify red and white pulp in the low magnifications, and to show that the high magnifications are white pulp. We have also rewritten the figure legend and Results and Discussion to clarify the significance of the findings.

(C)
1. The H&E images for p53WT do not correspond to the graphed data. The 4h graphed date shows more lipofuscin pigments and 24h less; the opposite is evident in the H&E images.
   - Thank you for pointing this out. In fact, while going back to the original files, we realised that the images had been switched during preparation of the figure. They are now in their correct position.

2. What was observed in white pulp regions of the spleen?
   - There was sometimes lipofuscin-like pigments in the white pulp of p53-mice, but much less than what was seen in the red pulp. We agree with the reviewer that this should be addressed, and have included this observation in the main text.

Level of interest: An article whose findings are important to those with closely related research interests
We have gone through the language, and corrected several errors (too many to list here). We have chosen to use UK-English grammar.

Statistical review:
Yes, and I have assessed the statistics in my report.

Declaration of competing interests:
I declare that I have no competing interests

Reviewer's report
Title:
Functional p53 is required for rapid restoration of daunorubicin-induced lesions of the spleen
Version:
1
Date:
21 March 2013
Reviewer:
Pankaj Trivedi

Reviewer's report:
The paradigmatic approach to p53 related cancer therapy suggests that wt p53 activity is desirable for optimal cancer therapy. In contrast, it has been reported that tumors with wt p53 presence might have worse prognosis. The use of DNA damaging chemotherapy has deleterious effect on normal cells also. Inasmuch as the normal cells would have wt p53, it is pertinent to ask what will be the effect of DNA damaging agents on normal cells.

The data presented in this MS reassure us that the initial cell death, apoptosis and the structure of the spleen in wt p53 bearing mice is recuperated after the withdrawal of the drug, while p53 null spleen suffered massive death. The data show that the presence of p53 is protective and restores DNA damage after withdrawal of the drug.

The paper is straightforward and clearly written, however I think there’s room for improvement.

Major Compulsory revisions:
A: The MS will improve if authors could show what happens to the p53 downstream (p21, mdm2 etc) genes in the two settings during the presence and the absence of the drug at various time points. It will clarify whether the protective effect of p53 presence is dependent/independent of these downstream genes in normal cells.

We agree with the reviewer’s suggestion. Accordingly, we have investigated the levels of p21, Bax, p63 and p73 during the DNR treatment of the mice. These data are presented in as a new Fig. 3, and in a new paragraph at the end of the Results and Discussion section. We believe that the information presented in this figure supplements the preceding data. Even though we are far from identifying the exact pathways involved in spleen lesions, we believe that Fig. 3 adds important information.

B: It would be interesting to silence p53 with siRNA in wt p53 spleen derived cells and see whether the findings in p53 null mice can be recapitulated here.
The second experiment suggested (isolate splenocytes and silence p53 with siRNA) is not possible for us to perform on a short notice for several reasons: First, we have to breed animals for the experiment, which can take up to several months. Secondly, based on our extensive experience in transfecting leukaemia cell lines, we know that this can be very difficult depending on the cell line. To perform this on isolated splenocytes, that already have been removed from their natural environment, needs optimization that can take several months, and still not be successful. Also, there is a concern with silencing protein translation with siRNA as there still can be some protein left that can respond to the drug treatment. We hope that the data presented, where p53 is knocked out completely, is sufficient to convince the readers of the role of p53 in anthracycline-induced cell death in non-cancer tissues.

Minor revisions: There are spelling and grammar errors in the MS which need to be taken care of.
This has been done, see comment to Reviewer 1 and editor.

Level of interest:
An article whose findings are important to those with closely related research interests

Quality of written English:
Needs some language corrections before being published.

Statistical review:
No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I have no competing interests.