Author's response to reviews

Title: miR-125b induces cellular senescence in malignant melanoma

Authors:

Anne Marie Rie Nyholm (rienyholm@gmail.com)
Catharina M Lerche (cler0001@bbh.regionh.dk)
Valentina Manfe (valentinamanfe@libero.it)
Edyta Biskup (edyta.biskup@gmail.com)
Peter Johansen (pjoh0809@gmail.com)
Niels Morling (niels.morling@forensic.ku.dk)
Martin Glud (glud.martin@gmail.com)
Robert Gniadecki (r.gniadecki@gmail.com)
Birthe Mørk Thomsen (ITHO0001@bbh.regionh.dk)

Version: 4
Date: 21 January 2014

Author's response to reviews: see over
To The Editor.

Please, find enclosed the revised version of the manuscript “miR-125b induces cellular senescence in malignant melanoma”, reference number MS: 6865590801043415.

The reviewers’ comments have been addressed and our responses are written below each comment:

Reviewer: Andor Pivarcsi

1. The observation about the relation of miR-125b expression to cell size is not entirely clear. First, most of the ISH-signal for miR-125b expression (Figure 1) seems to be localized in the cell nuclei rather than in the cytoplasm as it would be expected for a miRNA. How do the authors interpret this? Was the ISH signal specific in this experiment? U6 staining is shown as positive control on healthy skin, but not that of miR-125b. The staining should be performed on the same tissues both for the miRNA and for the controls.

The staining of the nuclei with miR-125b was consistent in all of our ISH and we have provided additional photographic documentation for that. We have also expanded on the interesting issue of the cytoplasmic staining which is indeed discernible for miR-125b in Fig. 1.

2. The staining intensity for miR-125b was very heterogeneous both in primary melanoma samples and in lymph node metastases. Unfortunately, the arrows on the figure do not clearly point to cells with particularly high miR-125b expression. Furthermore even cells with small cytoplasm expressed miR-125b at a high level. Cell size is difficult to assess without counterstain, which is difficult with ISHs. The authors could complement the figure with HE-staining on serial sections. Alternatively the authors could consider removing Figure 1 from the revised version of the manuscript, because it is not absolutely necessary for drawing the conclusion about the effect of miR-125b on melanoma cells.

The arrows have been placed more appropriately. We do not feel that the HE staining will further improve the validity of the data. Our slides are counterstained so the size of the cells is possible to appreciate. It is unfortunately technically impossible to combine full HE stain for HIS, since the blue haematoxylin color masks the IHS signal.

3. What was the transfection efficiency like in the miR-125b overexpression studies?

The transfection efficiency was not investigated since we have positively selected the transfected clones in selection media.

4. Please show standard deviation or standard error of mean for the results of the colony formation and proliferation assays obtained with the miR-125b overexpressing cells (Figure 2). If Figures would become too large, the authors could consider dividing it into two.

A graph showing the colony count and sd. has been added and the figure has been divided into 2 smaller figures.

5. The in situ hybridization results obtained with the mouse xenograft tumors reveal a different staining pattern as compared to that with the human tumors. In this case the staining is cytoplasmic, as
expected. However, miR-125b does not seem to be detectable in the control tumors in contrast to human tumors shown on Figure 1. What is the explanation for this?

The homogeneity of the tumors in figure 4 has now been commented in the results.

6. The ISH-picture of the control tumor on Figure 3A is completely blank. Tumor structure is not visible. Consider complementing this figure with HE-staining on parallel sections.

We do not have that problem with our Figure. This may be a resolution problem. Please write again if this is still a problem and we will try to enhance the contrast or send a high-resolution image.

Reviewer: Soheil S.S Dadras

Major:

1. In general, the data is very descriptive and in many places the results need to be quantified.

   The ISH is not easily quantifiable due to the fact that the intensity of the stain is not linearly correlated with miR expression. We have chosen not to quantify the HIS experiment; we consider such an approach rather misleading. All the rest of the data has been quantified.

2. Need to specific how many cases were tested for miR125b expression by ISH. There is no quantification of this data and it is unclear whether miR-125b is increased in nodal metastasis than the primary lesion in figure 1a and b. explain the location of ISH signal. Cytoplasmic or nuclear?

   As stated above we have not quantified the ISH data. The location has been explained and commented on in the manuscript.

3. What is the colony count for colony formation assay? Quantification?

   It is written in the text and a graphic presentation of the count has been added to figure 2.

4. Unclear what the molecular findings mean in the transfected cells, up-regualtion of P27, P53 and P21 mediated by miR-125b

   It is described in the introduction and the results how we interpret these findings.

5. Could the ISH results be confirmed by qRT-PCR in human tumor samples?

   We have previously published the phenomenon of down regulation of miR-125b in melanoma (Please see Glud M, Rossing M, Hother C, Holst L, Hastrup N, Nielsen FC, Gniadecki R, Drzewiecki KT: Downregulation of miR-125b in metastatic cutaneous malignant melanoma. Melanoma Res 2010, 20:479-484. PMID: 20827223). We have employed ISH not to re-quantify the data, but to provide the spatial expression pattern of this microRNA in human tumour samples.

6. The authors made definitive statements that miR-125b decreases amount of proliferation by measuring ki67 and cyclin D1. However, some cell based assay to measure the proliferation and invasion of the Mel-Juso cells when they are transfected by miRVec-125b and miRVec-control.

   We aim to investigate this issue in the future studies. Especially the idea to look at cell migration and invasiveness is very appealing.

7. Although this study was on miR-125b as a senescence inducer in malignant melanoma, in ISH assay for miR-125b, it is recommended to have nevus slides/samples from the patients as a control along with malignant melanomas, lymph node metastases, and superficial spreading MM.

   We employed ISH in this study to see if there could be a connection with senescence cells and an increased expression of miR-125b in MM. Senescence may operate via various mechanisms in various
tissues. The idea of including various types of melanomas at various stages, different types of naevi, etc is definitely interestingly and valid, but a bit off the scope and plans for this paper.

8. The introduction is missing some important description of latest miRNA characterization esp. by next generation sequencing in melanoma and other studies on miR-125, see below:


The introduction has been updated.

9. Since the authors showed only one image from their miR-125b ISH on human FFPEs. It is recommended to quantify the ISH results and be presented as a table/graph added to manuscript.

Please, see our response to your point 1.

10. In figure 3 authors, have put both ISH and IHC results together. It is recommended to either separate the results or explain the methodology in figure legend as Ki67 and cycline D expression was visually quantified using IHC.

It has been noted in the legend that the different stainings were made on different sections of the tumours.

Minor:

Label the figures more clearly and appropriately

The figures have been edited and legends has been rewritten

2. Page 9 last paragraph and last 2 lines: “Lungs and liver were taken out and cut in to three parts each, which were treated as the tumour samples”. Does author means these organs were checked for distance metastases?

Yes. It has been clarified.

All authors have approved the revised manuscript and agree with its submission to BMC Dermatology
We hope that these changes will be sufficient and hope to hear from you as soon as possible.

If you have any further comments or questions please do not hesitate to contact us.

Thank you for your time and consideration.

Sincerely,

Anne Marie Nyholm