Author's response to reviews

Title: MicroRNA in exosomes isolated directly from the liver circulation in patients with metastatic uveal melanoma

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Author's response to reviews: see over
Response to Reviewer 1

General comments: The manuscript “MicroRNA in exosomes isolated directly from the liver circulation in patients with metastatic uveal melanoma” by Eldh et al. presents new data on the presence of exosomes into the liver circulation in patients with metastatic uveal melanoma. Authors found that exosomes from the liver circulation of affected patients are enriched in some microRNAs, thus suggesting their potential use as diagnostic markers. The paper has a number of preliminary observations and the potential interest is hampered by some flaws mainly related to the paucity of samples. The paper can be considered for publication in BMC cancer pending the following revisions.

We thank the reviewer for a very positive general comment. In the first paragraph, the Reviewer suggest that the exosomal RNA that we have identified in the liver perfusates potentially could be utilized as diagnostic markers. We find this comment very helpful, but would like to emphasize that we have NOT validated the exosomal RNA from the perfusates as biomarkers, either diagnostic or prognostic. The aim was rather to determine whether exosomes could be isolated from a tumor bearing organ in vivo from human patients, with an attempt to determine whether the exosomes could be of tumor origin. We have therefore in current version of the manuscript removed ANY wording related to “potential future biomarker development” (Row 76-78, row 285-287 and row 345-348).

In the second paragraph, the Reviewer rightly suggest that the samples are very few, which certainly is an important limitation of the study. Importantly, there are no, or exceedingly few, surgical procedures that could allow isolation of exosomes directly from a tumor bearing organ in vivo in man, avoiding the huge dilution of vesicles in peripheral blood. Furthermore, there are exceedingly few centers globally that perform such procedure, and it certainly unlikely that any other such surgical center liaise with an exosomes focused research group.

Major points
1. Comment: For the clinical significance of the manuscript, it could be determinant to compare the miRNA profile of exosomes from metastatic patients with the miRNA profile of exosomes-derived from the peripheral blood of non-metastatic patients.

Response: The huge obstacle with this study, is as the Reviewer acknowledges, that a control perfusate in a healthy individual is unethical. As the Reviewer also suggest, a less attractive alternative would be to sample peripheral blood, but this study never had the aim to describe the profiles of melanoma-derived exosomes in peripheral blood. However, when we attempted that approach, we were not able to isolate sufficient quantities of exosomal RNA to perform qPCR. Importantly, this was already described in the original version of the manuscript (lines 264-296).

2. Comment: Authors presented data about the characterization of exosomes from liver perfusate but the characterization of peripheral blood- derived exosomes is missing.

Response: This comment by the Reviewer is very similar to major point No 1, and we would like to again emphasize the aim, to characterize the exosomes from the tumor-organ perfusates, and NOT peripheral blood.

Minor points:
1: In the discussion section authors should concentrate more on the discussion of the miRNAs that belong to cluster 3 that seems to be melanoma-specific.
Response: We fully agree with the Reviewer, that the significant similarities and differences in miRNA expression in exosomes from melanoma cell lines and our perfusate-derived exosomes are interesting. Importantly, there are no publications discussing miRs in cluster 3 in uveal melanoma making it hard to discuss these miRNA individually. We have however added a paragraph discussing them in general (row 306-316). We feel, and hope that the Editor agrees, that extending the already presented discussion further would be much too speculative, and if anything would weaken the Discussion.

2. Comment: line 236-239 (discussion section): authors say: Comparison of the exosomal miRNA profiles of patients and cell lines using cluster analysis revealed a clear relationship between the patient samples. Interestingly, the two melanoma cell lines did not cluster together, but did form a cluster together with the other cell lines”. Authors should discuss more this section. Why do they think that melanoma cells-derived exosomes have a microRNA profile more similar to other cancer cell lines (lung and breast) with respect to the exosomes isolated from the liver perfusion of metastatic uveal melanoma patients?

Response: Interestingly, patient material cluster better with patient material, and cell lines to cell lines. Thus, translating cell lines to clinical disease have important limitations, which perhaps are not surprising. However, the cell lines were the only appropriate controls we could consider under the ethical limitations of the study. We have now adapted the discussion in the lines above, to reflect this.

3. Comment: In material and method, line 103-104, authors say that “in addition to the liver perfusate, peripheral blood samples were taken prior to IHP and at 4 weeks, 3 months and 6-12 months after IHP”. In the results presented (fig. 3) they isolated exosomes from the peripheral blood prior to IHP showing data about the increase in circulating exosomes with respect to healthy control. No data are presented on exosomes isolated at other time points (4 week, 3 months, 6-12 months). What have been done with these samples? The analysis of extracellular vesicles before and after IHP could be significant.

Response: We thank the Reviewer hugely, as the inclusion of this information in the Methods is incorrect. In fact, we were unable to isolate specific exosomal RNA from the limited volume of blood samples. Thus, we have removed this text in the resubmitted version of the manuscript.

4. Comment: Line 299-300 (discussion section): authors say “The analysis revealed multiple miRNA clusters, with cluster 1 and 2 (Figure 4C) being similar among patients and four of the five melanoma cell lines”. In the manuscript is written that the melanoma cell lines used for the experiments are 2: A357 and MML-1, while from the sentence it seems that authors have considered five melanoma cell lines. Please correct.

Response: We thank the Reviewer for noticing this mistake, and of course we amend this in the resubmitted manuscript version.
Response to Reviewer 2:

1. Comment: Need to include controls for MelanA studies in Fig 2

Response: The proper control in this experiment would be exosomes isolated from a liver from a non-metastatic healthy individual, which unfortunately is prohibitive. For the Western blot, we have analysed exosomes from six individuals, and identified this protein surface marker conclusively in two patient samples.

2: References for vesicle RNA transfer exclude the previous work of Ratajczak and the comitant work of Aliotta and Deregibus. Please expand on the significance of this work - it appears marginal

Response: We have now included these references (row 56).

other revisions

3: Comment: Not clear the significance of the miRNA studies

Response: We thank the Reviewer for this comment, which we totally agree with. It would be beyond the scope of this study, however, to perform functional studies of the identified miRs.

4: Comment: Very small number of subjects

Response: One of the limitation of identifying exosomes in liver perfusates, is that the surgical procedure is exceedingly rare. It has taken us approximately four years to reach this point, and extending the numbers would unfortunately delay the publication with as many years. However, the material is really unique, and probably no other laboratory in the world would have the capacity to isolate exosomes from the local circulation of any organ with metastatic disease. We hope the Editor accepts the limitation of this study, but also acknowledge the uniqueness of the samples based on the rarity of the patients, and the interest this will reach within the uveal melanoma as well as exosomes communities.