Author's response to reviews

Title: MIA is a potential biomarker for tumor load in neurofibromatosis type 1

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Author's response to reviews: see over
Dear Dr. Mick Aulakh,

Thank you for reviewing our manuscript: “MIA is a potential biomarker for tumor load in neurofibromatosis type 1.” MS: 1924513230504997.

We were delighted to read the positive evaluation, and found reviewer comments very helpful. We responded to the comments and revised our manuscript accordingly. Please find below the “point by point response”. We are convinced that the manuscript was improved substantially and hope it can now be accepted for publication in journal BMC medicine.

Point-by-point response to comments of the reviewers
(the original comments are in italic black and our response in blue)

Reviewer: Rudi Beschorner
Minor essential revisions:

1. The numbers of cases/samples investigated in each group/subgroup is not always clear. Some times it seems that the numbers that are given in the text are not congruent with in numbers of cases/samples illustrated in the figures. Examples for such "obscurities" are the following:

These data should be checked and it should be more clearly stated (material and methods, results) how many cases (or how many blood samples from how many cases) were investigated each.

Response:
We now revised the data and corrected mistakes. We removed one patient from NF1 group as it was duplicated. Thus, MIA serum level was analysed in the cohort of 42 NF1 patients and 22 control healthy individuals.

- Fig. 3B: there are approx. 26 data points on patients with +pNF (n=17 in the text) and approx. 15 data points on -pNF patients (43 - 17 pNF -3 7 MPNST =19?).

Response:
Indeed the number 17 in the text was wrong. We thank the reviewer for pointing out this mistake. We corrected this and also added the numbers of patients in the legend of Fig.3

- p10: In the text the authors mention 10 cases with more than 100 subcutaneous
neurofibromas (n=10) but in figure 3C only 9 data points are visible in this group(s).

Response:
Indeed there are 9 patients in these groups. We corrected this mistake.

- Overall, in figures 3C (subcutaneous NF) and 3D (cutaneous NF) there are 39 and approximately 79 (!?) data points, respectively.

Response
The reviewer is right, Fig. 3D contained mistake which we now corrected. Both 3C and 3D contain 42 data points, but some of them may overlap with each other and thus not visible on the graphic. That may explain why only 39 could be counted.

- Figure 3E, right shows only 28 data points.

Response
The volumimetric data on the internal tumor load were only obtained for 30 out of 42 included NF1 patients. This was not clear in the previous version. We now pointed out clearly. On the graph, some data points overlap with each other, that’s why only 28 are visible.

2. The internal tumor load was determined on the basis of semi-automated volumetric measuring using MRIs. Patients were then divided in groups with no, low, moderate or high internal tumor load. The authors should briefly add information how these groups were separated (cut offs).

Response
This comment made us aware of an important point which we did not deal with correctly in the previous version of the manuscript. The original classification of patients was based on subjective estimation of one of the authors which did not always match the volumetric data. Now we defined the cut-offs clearly and objectively using the MRI volume data to <100, 100-350, 350-1000 and >1000ml. The differences in MIA serum level remain valid in this objective classification.

3. Immunohistochemistry (p9-10, Fig. 2): The authors state on page 10 that the proportion of MIA1 positive cells was about 1:1 in neurofibromas and up to 10:1 in MPNST and that "In general, MPNSTs exhibited higher cellular density and thus showed higher proportion of immunopositive tumor cells (Figure 2)."
However, a higher cellular density does not necessarily result in a higher proportion of positive cells. The figures show a higher cell density and a higher number of MIA1-positive cells in MPNST when compared to plexiform and non-plexiform neurofibromas. However, the (absolute) number of immunonegative cells is also higher in MPNST and the estimated proportion of immunostained cells seems to be approx. 50% in non-plexiform NF, approx. 70% in plexiform NF and approx. 50% in MPNST. Thus, if the figures already show representative results the statement in the section results needs to be corrected. Otherwise, the figures should be replaced and show roughly representative results.

Response:
Indeed, in the Fig. 2, MPNST gives the impression of higher MIA expression. However, carefully looking at the staining, one will find that this is because the high density of nuclei in MPNST. The
proportion is roughly that as the reviewer estimated. We corrected this in results and deleted the related statement in discussion.

In Results:
“…Proportion of MIA positive cells varies between 50 and 90 %. Most intensive staining was obtained in MPNST, which however, represents rather the high nuclei density. …”

We removed the statement in Discussion:
“Despite being expressed in MPNST, MIA serum level did not significantly differ in patients with and without MPNST. This may be explained by the small number of patients with MPNST in the present study, which did not allow detection of any significant difference.”

4. Figure 1B is not cited in the text (pleas add on page 10).

Response
The figure was now cited in the text on page 10.

5. Figure 3B: in the text a significant differences between cases with (+pNF) and without (-pNF) plexiform neurofibromas is stated (p=0.032). This should appropriately be illustrated in the figure.

Response
The missing labelling on the figure 3B was now added.

We thank Rudi Beschorner for reviewing our manuscript.

Reviewer: Bruce R Korf
1. Table 1 does not seem essential to include.

Response
We removed Table 1 and a detailed in-situ hybridization protocol from the methods sections and instead added in the Methods section reference:
“In-situ on paraffin sections were done according to standard protocol [9]”

2. What was the age range of patients? Were those with smaller tumor burdens generally younger?

Response
The age range of the NF1 patients was 14-72 years, we added this information in the results. We now did a correlation analysis and found indeed that more cutaneous tumors were found in older patients. For subcutaneous and internal tumors, we did not find such correlation. These data are also now given in the result section.

3. Could MIA positive and negative cells be distinguished morphologically or by special staining? For example, were positive (or negative) cells Schwann cells?

Response
The staining for MIA was visualized with chemical substrate on sections. Therefore, double labelling with S100 was not possible and we could not identify Schwann cells. However, based on histomorphological features for Schwann cells such as degenerative nuclear atypia, we deduce that MIA was both positive and negative in Schwann cell nuclei. We added this to the result section.
4. How was "internal tumor" defined? Did this category include plexiform neurofibromas, spinal neurofibromas, internal nodular neurofibromas?

**Response**
Yes, the category included all of them. However, lesions smaller than 3 cm in the longest diameter on MRI could not be ascertained as tumors and were not included. Many possible spinal tumors were not included neither because whole body MRI does not have the resolution to identify these small tumors. We revised in the methods accordingly.

5. Did the number of dermal tumors correlate with number of internal tumors?

The statement at the bottom of page 11 ("Analogically [sic] to its role in melanoma...") is speculative and not supported by data.

The number of dermal tumors did not correlate with internal tumors, but the number of subcutaneous tumors did. We revised the results accordingly.

“…, linear regression analysis revealed an association between total internal tumor load and number of subcutaneous tumors (P-value of 8.19E-17 for the F test), but not between internal tumor load and number of cutaneous tumors…”

We deleted the “Analogically” and modified the discussion.

We thank Bruce R Korf for reviewing our manuscript.