Author's response to reviews

Title: Neuropilin-2 induced by transforming growth factor-beta augments migration of hepatocellular carcinoma cells

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Version: 2
Date: 4 September 2015

Author's response to reviews: see over
Dear Prof. Solera,

Many thanks for your efficient handling of the manuscript MS: 1574699735171378 entitled “Neuropilin-2 Induced by Transforming Growth Factor-β Augments Migration of Hepatocellular Carcinoma Cells” by Philipp Wittmann, Markus Grubinger, Christian Gröger, Heidemarie Huber, Wolfgang Sieghart, Markus Peck-Radosavljevic and myself which we herewith submit again in revised form for publication as a research article in “BMC Cancer”.

We would like to thank the editorial for the very useful comments and the effort so far. We closely followed all suggestions of the reviewers and introduced corresponding changes into the text. For your convenience and to specify the revisions in the manuscript more clearly, the changes are highlighted in the following way: yellow + underline indicates new text, whereas yellow + strikethrough indicates deleted passages.

In the following, please find a detailed point-by-point discussion of the changes introduced into the text and comments to the issues raised by the reviewers.

**Notes to the Editor’s comment:**

As suggested by the editor, we provided the E-mail addresses of all authors in the title page and placed the Abstract on a separate page.

**Notes to the comments of reviewer 1:**

**To comment 1:**

The referee points out that only about half of grade 2, and about half of grade 3 HCCs have NRP2 expression, while a smaller but important percentage of grade 1 HCCs also have NRP2 expression. What about normal liver? Is there NRP2 expression in normal liver? What is the percentage of normal livers that express NRP2. This information is critical to our determining if NRP2 is really important in HCC, and if it may one day be a useful biomarker in HCC.

Thank you for this comment. In accordance, we included new text into the “Results” section stating that normal liver did not display NRP2 expression (page 8). We added a new reference as this observation confirmed recent findings (Wild et al, 2012).

**To comment 2:**

The reviewer notes that the in vitro studies do suggest a function for NRP2 in HCC. But the authors cause confusion regarding the exact relation of NRP2 to TGF-beta 1 signaling. Is NRP2 dependent on or independent of TGF beta 1 signaling? This needs to be clearly established, and made crystal clear in their discussion.

We appreciate this request and incorporated corresponding notions into the “Discussion” section which clarifies the relationship between NRP2 and TGF-beta signaling (page 11 and...
We state in the revised version that the diminished migration of HCC cells was similar either after reducing NRP2 levels or after blocking TGF-β signaling. We emphasize that TGF-beta inhibition on its own reduced NRP2 expression but could not completely block it, as the remaining NRP2 levels might allow cells to better migrate as compared to those cells with strongly reduced NRP2 levels after RNA interference. Yet, if HCC cells were blocked for both NRP2 and TGF-β signaling, a further decrease in HCC cell migration was observed. This additive operation of NRP2 and TGF-β signaling suggests that TGF-β additionally affects cell migration that is independent of NRP2.

To comment 3:

The referee states that confocal immunofluorescence is not quantitative, and therefore, not a suitable method to generate the data in figure 4.

Thank you for this suggestion. We completely agree that the confocal immunofluorescence analysis (IF) is qualitative rather than quantitative. We aimed to analyze the TGF-beta-mediated nuclear translocation of Smad2/3 and its possible dependence on NRP2 expression using the confocal IF. Importantly, this analysis confirms the quantitative data of phospho-Smad2 levels as determined by Western blotting (Figure 4A and 4B). Thus, the data obtained by two independent methods (confocal IF and WB) underline that the canonical TGF-beta signaling is not affected by NRP2 expression. We believe that this is sufficiently described in the “Results” section and hope for the understanding of the referee that no changes were introduced into the text.

Notes to the comments of reviewer 2:

To comment 1:

The reviewer notes that although the experiments appear to be thoughtfully planned it is difficult to simply extrapolate the results from few in vitro experiments into the observations that were made in tumor specimens. A direct link between the two was not investigated through these experiments. The experiments described in the manuscript therefore should use the immunohistochemical NRP2 expression data from tumor specimens as supportive data that cannot be used in conjunction with in vitro data.

Thank you for this comment. In accordance with your suggestion, we state in the revised version that the NRP2 expression observed by immunohistochemical analysis was found in de-differentiated mesenchymal-like HCC cells in vitro which supports the idea that NRP2 expression correlates with a de-differentiated phenotype (page 9). Thus, the term “confirms” was replaced with “supports” in order to put the link between data obtained in cell culture and those obtained in patients into perspective.

To comment 2:

The referee states that also for any cell migration based in vitro investigations it is always prudent to use several parallel assays to prove the same hypothesis, which is missing in this manuscript. The authors did not provide a detailed procedure for conducting the wound healing assays, such as how the wound measurements were done and at how many points within the wound were measured to get an average data. The manuscript also lacks the detailed procedure for RQ-PCR, control gene expression that used to normalize the RQ-PCR data and the type of comparison that done among epithelial and mesenchymal making it difficult to understand and make sense out of Figure 1D.

Many thanks for this suggestion. We agree and introduced new text into the “Methods” section which describes the procedures for both wound healing assays and RQ-PCR in more detail.
detail (pages 6 and 7). With respect to the use of parallel assays to prove the same hypothesis, we would like to underline that the migration of HCC cells was analyzed by both Transwell as well as wound healing assays. To make this point more clear for the reader, we incorporated additional data showing the quantitative evaluation of wound healing assays (Figure 2D). A corresponding notion was included into the Legend to Figure 2 (page 17).

**To comment 3:**

The reviewer notes that it was not clear from the manuscript text whether the experiments were performed once in triplicates or the data presented in the manuscript is from three independent experiments with at least 3 replicates in each experiment.

Thank you for this notion. We closely followed this request and provided information about the number of experiments that were performed. Corresponding statements were incorporated into the Legends to Figure 1, 2, 3 and 4 (page 17 and 18).

Overall, we responded to all issues raised by the reviewer and we believe that we could sufficiently answer all requests. With the inclusion of suggested intext revisions, we are convinced that the manuscript is significantly improved. We hope that you find our revised work suitable for publication in “BMC Cancer”.

Looking forward to your evaluation, I remain

Yours sincerely

Wolfgang Mikulits, Ph.D.