Author's response to reviews

Title: Increased MiR-221 Expression in Hepatocellular Carcinoma Tissues and Its Role in Enhancing Cell Growth and Inhibiting Apoptosis in vitro

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Author's response to reviews: see over
Dear Editor:

We would like to express our appreciation to you and the reviewers for suggesting how to improve our paper. Here are our replies to the comments from the referees. Revised portions are marked in red in the new manuscript. One final version without marks will be uploaded via “Additional material files”.

Two new figures were added, so the order of figures had been changed.

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Reviewer 1

1.1 Since this concerns a study on archival material the authors may have information on patient survival. If possible please provide this data.

Reply: Since the cases involved in the current study are from 2010–2011, we don’t have the “overall survival” data for all the patients. Although “disease- and progression–free survival” are appropriate endpoints in other solid tumors, they are particularly unreliable endpoints in HCC research because death could result from the natural history of cirrhosis. That is, a type II error might result from using “progression–free survival” as an endpoint in a suboptimal population (Llovet JM, et al. J Natl Cancer Inst 2008, 100:698–711). However, we collect the “recurrence–free” data for some patients. Forty–eight among 76 patients were followed up till 6th, July, 2012. Time–to–recurrence was collected from randomization (operation date) to radiological recurrence. Time–to–recurrence for all 48 cases was 28.94 ± 3.20 weeks. The patients with high expression of miR–221 (higher than the median level) had a shorter time–to–recurrence compared to those with low expression (24.15 ± 3.11 vs 33.73 ± 5.48 weeks), however, the difference is not significant (P=0.129). A larger sample
size and longer following up are required to investigate the relationship between miR–221 level and the recurrence and patients’ overall survival. Extra figure and texts were added to the new manuscript.

1.2 Microscopic vaso-invasion has predictive value towards survival. These data is not provided, if available please provide.
Reply: The microscopic vaso-invasion data are available. The results were added in Table 1, also in the result part.

1.3 The text is at some points is too long. I suggest to delete: in Background section the paragraph related to Doxorubicin and Cisplatin/IFN therapy. These therapies are obsolete and do not need to be discussed. Also delete on page 6 the paragraph starting with ‘After transfection with…………(data not shown)’. This is shown in the figures and there is no need to describe this in detail. Finally delete in Discussion (page 7 and 8) the entire paragraph starting with ‘Fromalin-fixed and……RT-qPCR procedure’. It is well known that miRNA survives formalin-fixation and this does not have to be repeated here.
Reply: Thank you for the suggestions and the texts (point1 and point 3) mentioned above were deleted. But for point 2 on page 6, ΔΔCq was not shown in any figures. Another reviewer also suggests that the transfection efficiency should be described. Can this part be kept?

1.4 Throughout the text there are several grammatical errors. Please correct.
Reply: They were corrected and the revised manuscript has been reviewed by two professors from Belgium.

Reviewer 2

2.1 Authors should provide data for transfection efficiency.
Reply: To determine the transfection efficiency of the transfection reagent in current study, we first tested HepB3 cells transfection efficiency with two approaches: cell fluorescence by siGLO Transfection Indicators and cell death induced by TOX Transfection Control. The transfection efficiency was higher than 89% at 72 hrs and 94% at 96 hrs, as assessed by either CellTiter-Blue® Cell Viability Assay, or fluorescence, as assessed by fluorescence microscopy. These data suggested that the transfection efficiency was nearly optimal with the current method for transfection. These texts were added to the new manuscript. Afterwards, transfection efficiency of miR–221 mimic and inhibitor was further verified by RT-qPCR assay. The miR–221 expression level decreased with miR–221 inhibitor and increased with miR–221 mimic with a time-dependent manner, in different degrees. After transfection with the miR–221 inhibitor, the biggest ΔΔCq was 2.13 (77.15% knock-down) for HepB3, 1.32 (59.95% knock-down) for HepG2 and SNU449 1.78 (70.88% miR–221 knock-down) 96 hrs post-transfection. After transfecting the miR–221 mimic for 96 hrs, miR–221 levels were most severely increased, with ΔΔCq –13.78 (14065.74 folds upregulation) for HepB3, –12.44 (5555.65 folds upregulation) for HepG2 and –11.97 (4010.71 folds upregulation) for SNU449.
2.2 Further, to confirm successful transfections, authors should show altered protein levels of one of the established miR-221 targets such as p27.

Reply: Extra experiments on the protein levels of CDKN1B/p27 and CDKN1C/p57 were performed using western blot. Indeed, downregulation of both CDKN1B/p27 and CDKN1C/p57 were observed when miR-221 mimic was transfected into HepB3, HepG2 and SNU449 cells. An upregulation of these proteins were also seen after the transfection of miR-221 inhibitors. Texts and one figure of HepB3 with miR-221 transfection were added.

2.3 Authors demonstrate that miR-221 mimic increases proliferation while inhibition of miR-221 decreases proliferation. Authors should cite previous published reports, which also show the role of miR-221 in hepatocyte proliferation during liver regeneration.

Reply: The following reference and some texts were added.


2.4 Similarly, previous reports of role of miR-221 in FAS-induced apoptosis and TNF alpha-induced apoptosis should be cited.

Reply: The following references and some texts were added.


2.5 In Figure 8, authors show that miR-221 mimic mediated protection against apoptosis is significant in HepB3 cells but not in HepG2 and SNU449 cells. A potential explanation is differential expression of endogenous miR-221 expression among different cell lines. Furthermore, previous reports have also shown that miR-221 mimic protects against apoptosis. Therefore, authors should transfec miR-221 mimic at higher concentrations and analyze its effect on apoptosis in HepG2 and SNU449 cells.

Reply: We showed that miR-221 mimic mediated protection against apoptosis is significant in HepB3 cells, but with only about 20% reduction, and the reduction of apoptosis was even less in HepG2 and SNU449 cells (around 10–15%). We do agree that the expression of the endogenous level of miR-221 expression is slightly different in these 3 cell lines, and the
upregulating level also varies after mimic transfection, as measured by the real-time RT-qPCR. However, the effect of miR-221 mimic on apoptosis in all the 3 cell lines was actually weak. The concentration of miR-221 mimic used was 200nm, already a high dose compared to literatures. We did try to increase the concentration to 300nm, unfortunately, no significant influence was observed in HepG2 and SNU449 cells. We think there could be a saturation threshold in these cell lines by a single miRNA mimic. Some texts were added in the manuscript.

Reviewer 3
3.1 The authors have to address the definition of "TNM" stages and explain whether TNM stages correlate with patients outcomes.
Reply: The TNM stages used in the current study was based on the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) staging system. The new 7th edition (TNM-7) of the AJCC/UICC TNM system was introduced in 2009. This classification considers tumor size and number, vascular invasion, bilobar involvement and extra-hepatic metastasis. Some texts were added to the new manuscript.

3.2 The authors find that the miR-221 expression levels are correlated with clinic pathological parameters, especially with status of tumor capsular infiltration. However, they fail to demonstrate the effects of miR-221 in altering capsular infiltration status and whether clinic pathological parameters have prognostic values. A detail mechanism study is required to enhance novelty of entire study.
Reply: In the current study, we showed that the relative expression is related to the progression of HCC and miR-221 over-expression was related to the status of tumor capsular infiltration. Functionally, the miR-221 inhibitor inhibited cell growth, arrested cell cycle in G1/S-phase and increased apoptosis in vitro. In the revised version, we added data of the relationship between recurrence and miR-221 levels in clinical study, also the data of microscopic vaso-invasion (see above the replies to the first reviewer), which further confirms that expression of miR-221 in FFPE tissues could provide predictive significance for recurrence and prognosis of HCC patients. And we also performed additional in vitro experiments to examine the possible targets of miR-221 in HCC cells (see above the replies to the second reviewer). The results showed that in these 3 cell lines studied, CDKN1B/p27 and CDKN1C/p57 are proved to be the targets of miR-221.

Looking forward to your reply and thank you very much considering our manuscript for publication in your journal.

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