Format for ANSWERING REVIEWERS

November 28, 2016

Dear Editor,

Thank you very much for the consideration of our manuscript "Hsa-mir-183 is frequently methylated and related to poor survival in human hepatocellular carcinoma". We are very pleased about the overall positive evaluation of our manuscript ("interesting, clearly explaining epigenetic regulation of miR-183 in HCC and its potential role as a novel surrogate and prognostic marker, priority for publishing, excellent") and appreciate all comments raised by the reviewers, which give us the possibility to improve our manuscript. We thank for the opportunity to resubmit a revised version addressing all comments from the reviewers. A point by point-reply describing all changes made in detail is included. All changes in the text are highlighted in yellow.

Please find enclosed the edited manuscript in Word format (file name: WJG_30486 revised).
Title: Hsa-mir-183 is frequently methylated and related to poor survival in human hepatocellular carcinoma
Authors: Sumadi Lukman Anwar, Till Krech, Britta Hasemeier, Elisa Schipper, Nora Schweitzer, Arndt Vogel, Hans Kreipe, Reena Buurman, Britta Skawran, Ulrich Lehmann

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The manuscript has been improved according to the suggestions of reviewers:
Reveiwer #1 (**00006931)**
Comment to author:

1. In methods section and in table 2, authors mentioned 10 samples with HCAs but in results section on page 7 about DNA methylation analysis in benign liver tumors and healthy liver tissues authors mentioned 15 cases of HCAs. Please revisit this discrepancy and also include the information about the healthy liver tissues (n=5) (their source, characteristics and how they obtained) in the Table 1.
   Answer: In total, we included 15 benign liver tumors in this study consisting of 10 HCAs and 5 FNHs. We have made corrections as shown at the revised manuscript Result section page 11. The healthy liver tissues (n=5) were collected from autopsy of patients other than liver diseases (without history of hepatitis, obesity, and diabetes) and the liver tissues have been confirmed by 2 independent pathologists as healthy livers.

2. Authors did three sequential transfections in HLE cells to knock down DNMT. How was the cell viability after knocking down DNMTs?
   Answer: We performed 3 sequential DNMTs-knockdown experiments, including a scrambled oligonuclotides as well as non-treated cells as negative controls. After first knockdown, the cells were plated at 24-well plate, and after second and third

knockdown, the cells were re-plated in 12-well plates and 6-well plates, consecutively. We observed that after knockdown, the cells were still viable and grown as we need to transfer into bigger wells. As we included scramble siRNAs and non-treated cells in the plates during all experiments, we could make sure the viability of the cells among those treated with DNMTs-siRNAs, scramble-siRNAs and non-treated (please see page 8 revised version).

3. Authors described mRNA quantification on page 6 but it's not clear which mRNA expression was determined in the manuscript.
   Answer: We used real-time PCR-based mRNA quantification to check the levels of DNMT1, DNMT3A, and DNMT3B transcripts after DNMT-knockdown in addition to western blotting. For quantification, we used the following pre-developed TaqMan assays from ThermoFisherScientific (Dreieich, Germany): Hs00154749_m1 for DNMT1, Hs01027166_m1 for DNMT3A, and Hs00171876_m1 for DNMT3B. For transcript normalization GADPH and GUSB mRNAs were measured. This is now explained in more detail in the revised version of the manuscript at page 9.

4. Please correct the sentence on page 9 in the discussion section 'since microRNAs modulate several microRNAs and transcription factors' to since microRNAs modulate several mRNAs and transcription factors.
   Answer: Done as suggested (page 14).

5. In Figure 1, miRNA expression is shown after knocking down DNMT in cell line. Please show the methylation level of miR-183 promoter in the same figure as a different panel to conclude methylation-mediated regulation of this miRNA.
   Answer: Done as requested. We add Figure 1b and revised the legend accordingly. The relationship between DNMT knock down, promoter methylation and microRNA expression is now explained in more detail in the "Results" section. (Please see page 11 of the revised version).

6. The authors need to discuss their findings to reveal the mechanistic details about miR-183 methylation and the associated impact on cell proliferation and HCC development. Similarly miRNA targets should be validated in tumor tissues. There should be a correlation between miRNA silencing by methylation, higher expression of target mRNA and increased HCC progression before concluding with poor survival in HCC.
   Answer: We have discussed the effects of differential DNA methylation to the expression of miR-183 as well as the mRNA targets and their biological pathways related to hepatocarcinogenesis. However, association of miR-183 silencing through hypermethylation, higher target mRNA expression, hepatocarcinogenesis and poor survival is a complex process involving diverse biological and clinical variables including tumor stadium, treatment, comorbidity, HCC subtypes, tumor grade, and not only a direct straightforward correlation between DNA methylation and HCC survival. In this study, we elaborated on the role of DNA methylation aberrations of microRNAs in hepatocarcinogenesis. Hypermethylation of miR-183 might not be able to replace other prognostic factors to predict HCC survival, but offers an

additional relevant marker to determine HCC prognosis. The "Discussion" section was amended accordingly (see last paragraph).

7. In supplementary figure 2, methylation level of hsa-miR-23 is shown in clinical samples (panel A) and hsa-miR-25 level in cell lines (panel B). Why the hsa-miR-25 methylation is not determined in clinical samples as in panel A? The authors please discuss
   Answer: Hypermethylation at the promoter of *hsa-mir-25* cluster was not observed in 7 HCC cell lines (HLE, HLF, Huh7, HepG2, SNU182, and SNU387) as well as in healthy adult liver epithelial lines (THLE2 and THLE3) indicating that the miR-25 upregulation after *DNMT* knockdown is likely due to indirect effects. Therefore, we did not further analyse miR-25 methylation in clinical samples. On the other hand, differential methylation has been observed at the promoter of *hsa-mir-23* in our HCC cell lines, therefore we further analyzed clinical samples. The manuscript has been revised accordingly (please see page 11).

Reviewer #2 (**02997239)**

1. The pathological criteria of the studied specimens should be presented.
   Answer: Established pathological criteria were used by our 2 experienced pathologists examining the specimens included in this study independently to provide accurate diagnosis, including tumor size and localization, growth pattern of development, penetration to liver capsule, resection border, extrahepatic spread) as well as microscopic features (including grade of differentiation, vascular invasion, lymph node metastasis, Milan criteria for low and high risk for tumor progression). The liver tumor classification according to 2010 WHO classification was used (IARC).
   The "Materials and Methods" section has been amended accordingly, providing also in the revised version all relevant references.

2. Impact of deregulated studied microRNAs and DNA methylation on pathological criteria of studied specimens.
   Answer: Done as suggested. Our study focused on differential DNA methylation of microRNAs in hepatocellular carcinoma in comparison with benign liver tumor (HCAs, FNHs) and healthy liver tissues. Differential DNA methylation of miR-183 are commonly found in HCCs, but not found in benign liver tumors as well as in healthy liver tissues. Therefore, we mentioned that hypermethylation of miR-183 is a good candidate for a diagnostic marker to differentiate malignant liver tumor from benign lesions especially in cases where relying in histopathology are difficult for example in distinguishing between HCA and well-differentiated HCC (please see result and discussion, page 12 and 14).

3. The discussion is in need to focus on hepatocellular carcinoma and the studied

microRNAs.
Answer: Done as suggested. We add discussion in the first paragraph focusing on miR-183 differential methylation and HCC and the last paragraph discussing role of aberrant DNA methylation in microRNA promoters in HCC (page 15).

4. Minor language changes should be done
Answer: Done as suggested

Thank you again for considering our manuscript for publication in the *World Journal of Gastroenterology.*

Sincerely yours, I should also sign here. And my address should be added.

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