Format for ANSWERING REVIEWERS

July 15th, 2016

Dear Editor,

Please find hereby the edited manuscript in Word format (with file name: 26795-Revised manuscript.docx).

Title: Circulating predictive and diagnostic biomarkers for Hepatitis B Virus-associated hepatocellular carcinoma

Authors: Stijn Van Hees, Peter Michielsen, Thomas Vanwolleghem

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 26795

The manuscript has been adapted according to the suggestions of the reviewers and the editor:

1. Revision has been made according to the suggestions and questions raised by the reviewers
   1) Referee 1
      No questions
   2) Referee 2
      No questions
   3) Referee 3

   Question 1: For each biomarker, please specify whether they are liver specific and liver cancer specific proteins; if not, what other cell types and/or cancers can contribute. The authors did this for some of the biomarkers but not all.
   Thank you for this question. The necessary information was added to the manuscript and highlighted yellow. We kindly refer to sentence 4 of the AFP part, sentence 3 of the DCP part, sentence 1-2 of the osteopontin part, sentence 1 of the GP73 part, sentence 3 of the GPC3 part, sentence 2 of the SCCA part, sentence 2 of the DKK1 part and to sentence 4 of the miRNA part. All added information was highlighted yellow.

   Question 2: For each biomarker, please highlight the most reliable method for detection of the specific biomarker and add a summary column for detection method in table one with citation.
   Thank you for your question. The required information was added to the manuscript and highlighted yellow. The choice of the most reliable method was based on recent studies involving at least 100 patients.

   Question 3: In AFP-13 section, the sensitivity ranges from 36%-96% which is a quite wide range. Please explain/discuss the reason for such a high range among different studies and highlight the data from some rigorous studies.
   Thank you for this question. The following part was added to the manuscript and highlighted yellow:

"However these studies assessing the clinical potential of AFP-l3 use different cut-off levels, test methods and patient numbers, resulting in a wide range of detected sensitivity. A study from 2009 measuring the fraction of AFP-l3 to total AFP using an automated immunologic analyzer and a cutoff of 10% AFP-l3 to total AFP in 419 HCC patients and 417 cirrhotic controls, found a sensitivity of 42% to detect HCC.[1] AFP-l3 fractions were measured using Western blotting in another study, involving 388 HCC patients and 212 controls with a cutoff of 15% AFP-l3 to total AFP, resulting in a sensitivity of 21%.[2]

Question 4: It's interesting to see miRNA as promising biomarkers for HCC. Please add more details about the functions and downstream targets/signaling for miRNA-21 and miRNA-122, and highlight some studies on them in HCC.

Thank you for this question. The following, highlighted information was added to the miRNA paragraph.

"miRNA-21 inhibits tumor suppression by inhibiting tumor suppressor pathway (e.g. ATK and MAPK) activating phosphatases, whereas miRNA-122 inhibits tumor growth by acting as a tumor suppressor gene."

Question 5: There are several studies on circulating tumor cells for HCC. Please review some of these studies and discuss whether this could be useful for detection of HCC.

Thank you for this question. There are indeed some studies on the role of circulating tumor cells in HCC. However, most of these studies focus on prognosis and prediction of disease progression after HCC diagnosis and thus do not fit within the scope of our review. The following part was added to the paragraph entitled 'other diagnostic biomarkers' of the manuscript and highlighted yellow:

"Several studies have been published on circulating tumor cells for HCC. However, most of published studies focus on prognosis after HCC diagnosis and prediction of disease progression rather than on the diagnosis of HCC.[3-5]"

Question 6: In discussion, there is a brief paragraph about SNP, please discuss both germline and somatic gene mutation analysis that could link to HCC, and the usage of gene mutation for detection and prediction for HCC development.

Thank you for this question. Indeed, both germline and somatic SNP's have been linked to HCC. Therefore, the following part was added to the manuscript and highlighted yellow.

"Increasing evidence indicates that SNP's in the STAT4, MDM2 and HFE gene, determined on whole blood, are germline risk factors for HCC.[6, 7] On the other hand, also somatically acquired mutations, e.g. in the TP53 gene, have been associated with an increased risk for HCC.[8] All together these findings are strongly suggestive for interindividual differences in the genetic predisposition for HCC development, a predisposition that can be boosted by additional somatic mutations."

Question 7: In discussion, last paragraph, please discuss in more details the systems biology techniques that can be applied on liver tissue to predict HCC development with citations.

Thank you for this question. Besides proteomics, also genomics have been successfully applied on liver tissue to predict HCC development in a hepatitis C virus-infected liver. The following part was added to the manuscript and highlighted yellow:

"Other groups have focused on genomics and have identified a gene signature in liver tissue of Hepatitis C virus infected patients predictive of HCC development.[9, 10] It could be of interest to identify corresponding secretory biomarkers in blood."

2. The manuscript was updated according to the Guidelines and Requirements for Manuscript Revision-Topic Highlight. Copies of the figure in decomposable and editable format are attached in PowerPoint format (file name: 26795_Figure_submission.pptx). All tables included in the manuscript are now editable.
3. An Audio Core Tip was added during the re-submission process (File name: 26795-Audio core Tip.mp3)
4. The manuscript was subjected to CrossCheck analysis and the final title to Google Scholar. PrintScreens of both processes are attached (File name: 26795-Google Scholar.pdf and 26795-CrossCheck.pdf).
5. A conflict-of-interest statement was drawn up and is attached (File name: 26795-Conflict-of-interest statement.pdf)
6. The Letter of Approval of the stated Grant was added (file name: 26795–Grant application approval.pdf).
7. The final version of the manuscript was critically reviewed and edited for proper use of English and English grammar by the head of our laboratory, prof. dr. B de Winter (in possession of CEFR level C1; file: 26795-Language certificate.pdf). Her effort has been acknowledged in the 'acknowledgement' part of the manuscript. All language changes are marked in the manuscript using the 'track changes' option in Word.
8. The Copyright Assignment was signed and is attached. (File name: 26795-Copyright assignment.pdf)

We thank the reviewers for their comments and hope our revised paper is acceptable for publication in the *World Journal of Gastroenterology*.

Yours sincerely,

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References

1 Marrero JA, Feng Z, Wang Y, Nguyen MH, Befeler AS, Roberts LR, Reddy KR, Harnois D, Llovet JM, Normolle D, Dalhgren J, Chia D, Lok AS, Wagner PD, Srivastava S, Schwartz M. Alpha-fetoprotein, des-gamma carboxyprothrombin, and lectin-bound alpha-fetoprotein in early hepatocellular carcinoma. *Gastroenterology* 2009; **137**(1): 110-118 [PMID: 19362088 PMCID: Pmc2704256 DOI: 10.1053/j.gastro.2009.04.005]

2 Oka H, Saito A, Ito K, Kumada T, Satomura S, Kasugai H, Osaki Y, Seki T, Kudo M, Tanaka M. Multicenter prospective analysis of newly diagnosed hepatocellular carcinoma with respect to the percentage of Lens culinaris agglutinin-reactive alpha-fetoprotein. *Journal of gastroenterology and hepatology* 2001; **16**(12): 1378-1383 [PMID: 11851836]

3 Fan JL, Yang YF, Yuan CH, Chen H, Wang FB. Circulating Tumor Cells for Predicting the Prognostic of Patients with Hepatocellular Carcinoma: A Meta Analysis. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2015; **37**(2): 629-640 [PMID: 26344495 DOI: 10.1159/000430382]

4 Kelley RK, Magbanua MJ, Butler TM, Collisson EA, Hwang J, Sidiropoulos N, Evason K, McWhirter RM, Hameed B, Wayne EM, Yao FY, Venook AP, Park JW. Circulating tumor cells in hepatocellular carcinoma: a pilot study of detection, enumeration, and next-generation sequencing in cases and controls. *BMC cancer* 2015; **15**: 206 [PMID: 25884197 PMCID: Pmc4399150 DOI: 10.1186/s12885-015-1195-z]

5 Yan J, Fan Z, Wu X, Xu M, Jiang J, Tan C, Wu W, Wei X, Zhou J. Circulating tumor cells are correlated with disease progression and treatment response in an orthotopic hepatocellular carcinoma model. *Cytometry Part A : the journal of the International Society for Analytical Cytology* 2015; **87**(11): 1020-1028 [PMID: 26355643 DOI: 10.1002/cyto.a.22782]

6 Zhang L, Xu K, Liu C, Chen J. Meta-analysis reveals an association of STAT4 polymorphism with hepatocellular carcinoma risk. *Hepatology research : the official journal of the Japan Society of Hepatology* 2016 [PMID: 27126090 DOI: 10.1111/hepr.12733]

7 Jin F, Xiong WJ, Jing JC, Feng Z, Qu LS, Shen XZ. Evaluation of the association studies of single nucleotide polymorphisms and hepatocellular carcinoma: a systematic review. *Journal of cancer research and clinical oncology* 2011; **137**(7): 1095-1104 [PMID: 21240526 DOI: 10.1007/s00432-010-0970-0]

8 Yao S, Johnson C, Hu Q, Yan L, Liu B, Ambrosone CB, Wang J, Liu S. Differences in somatic mutation landscape of hepatocellular carcinoma in Asian American and European American populations. *Oncotarget* 2016 [PMID: 27246981 DOI: 10.18632/oncotarget.9636]

9 King LY, Canasto-Chibuque C, Johnson KB, Yip S, Chen X, Kojima K, Deshmukh M, Venkatesh A, Tan PS, Sun X, Villanueva A, Sangiovanni A, Nair V, Mahajan M, Kobayashi M, Kumada H, Iavarone M, Colombo M, Fiel MI, Friedman SL, Llovet JM, Chung RT, Hoshida Y. A genomic and clinical prognostic index for hepatitis C-related early-stage cirrhosis that predicts clinical deterioration. *Gut* 2015; **64**(8): 1296-1302 [PMID: 25143343 PMCID: Pmc4336233 DOI: 10.1136/gutjnl-2014-307862]

10 Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, Gupta S, Moore J, Wrobel MJ, Lerner J, Reich M, Chan JA, Glickman JN, Ikeda K, Hashimoto M, Watanabe G, Daidone MG, Roayaie S, Schwartz M, Thung S, Salvesen HB, Gabriel S, Mazzaferro V, Bruix J, Friedman SL, Kumada H, Llovet JM, Golub TR. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *The New England journal of medicine* 2008; **359**(19): 1995-2004 [PMID: 18923165 PMCID: Pmc2963075 DOI: 10.1056/NEJMoa0804525]