ANSWERING REVIEWERS

May 21, 2015

Dear Editor,

Thank you for your efforts regarding our manuscript entitled **"Biomarkers for the early diagnosis of hepatocellular carcinoma"** previously submitted to your journal.
The issues raised by the reviewers were carefully checked, and our manuscript was modified according to their suggestions. Our manuscript was provided comprehensive editing services by AmEditor, Inc.

Please find enclosed the edited manuscript in Word format (file name: 17659-review).

Title: Biomarkers for the early diagnosis of hepatocellular carcinoma

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 17659

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

Sincerely yours,

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Reviewer 1

Major comment:
There is an important novelty issue:
Schütte K, Schulz C, Link A, Malfertheiner P. Current biomarkers for hepatocellular carcinoma: Surveillance, diagnosis and prediction of prognosis. World J Hepatol. 2015 Feb 27;7(2):139-49.
⇨ Please provide an overview of the differences between the presented review and the supra recent reference.

Thank you for the information of supra recent reference.
The reference is different to our review which focuses on early diagnosis of HCC as to mention about prediction of prognosis and focus on microRNA particularly.
And we added this reference to [92]

Minors:
Abstract:
"Genomics and various other technological advances over"
⇨ Please don't mention only one general technique. Mention the most promising biomarkers, which is most likely to get clinically validated in the near future. Rephrase this sentence.

We agree with this comment, so we rephrased the following sentence on page 3, lines 10.

Advances in genomics and proteomics platforms and biomarker assay techniques over the last decade have resulted in the identification of numerous novel biomarkers and have improved the diagnosis of HCC. The most promising biomarkers, such as glypican-3, osteopontin, Golgi protein-73 and nucleic acids including microRNAs, are most likely to become clinically validated in the near future.

"In the present article, we provide an up-to-date review of biomarkers that are used for the early diagnosis of HCC."
⇨ Please rephrase this sentence.

We agree with this comment, so we added your informing supra recent reference to [92] and rephrased the following sentence on page 3, lines 18.

In this article, we provide an overview of the biomarkers that are currently used for the early diagnosis of HCC.

Introduction:
"HCC mostly develops in patients with a history of cirrhosis due to the current epidemic of non-alcoholic fatty liver disease and hepatitis C virus (HCV) infection, in which there is continuous

inflammation and healing in hepatocytes." 2

⇨ Please acknowledge the role of chronic alcohol abuse in HCC etiology.

We agree with this comment, so we added the following phrase on page 5, lines 6.

chronic alcohol abuse,

"Unfortunately, even this is inadequate, and very few HCC biomarkers have sufficiently high sensitivity for the detection of early stage HCC."
⇨ Reference?

Thank you for your comment.
Although several biomarkers which have high sensitivity, such as microRNA, have been identified in basic research, many of them have not used in clinical practice yet.
So we rephrased the following sentence on page 5, lines 29.

Unfortunately, even this approach is inadequate, and very few HCC biomarkers demonstrate sufficient diagnostic performance for early stage HCC in clinical practice.

Please acknowledge the importance of development of novel chemopreventive strategies during HCC surveillance of cirrhosis patients in your introduction.

We agree with this comment, so we added the following sentence on page 5, lines 12.

Furthermore, such biomarkers may influence the development of novel chemopreventive strategies for use during HCC surveillance of patients with cirrhosis.

Please discuss the relevance of the sensitivity versus specificity of a biomarker in general before discussing the various biomarkers.

We agree with this comment, so we added the following sentence on page 6, lines 4.

In general, a biomarker valuable for clinical use achieves a level of sensitivity and specificity of ≥ 90%, and is non-invasive and cost-effective to allow widespread use.

Please provide a title between introduction and the list of biomarkers.

We agree with this comment, so we added the following title on page 6, lines 20.

LIST OF HCC BIOMARKERS

List of HCC biomarkers:
"These results indicate that hs-AFP-L3% could be a useful biomarker for detecting early stage HCC. In the future, hs-AFP-L3 may be a useful biomarker for the early detection of HCC in clinical practice."
⇨ Please make one concluding sentence of these two.

We agree with this comment, so we rephrased the following sentence on page 9, lines 2.

These results indicate that hs-AFP-L3% could be a valuable biomarker for detecting early stage HCC and may be used for clinical practice in the near future.

"A large multi-center case-control study subsequently showed that DCP may have value for early stage HCC detection, with a sensitivity of **56**% in early stage patients[39]. To improve the insufficient sensitivity for early stage HCC detection, combined use with AFP was assessed. The combination of markers increased the sensitivity from **65**% to 87% 3 months prior to HCC diagnosis. However, the specificity decreased, from 84% to 69%."
⇨ Was the sensitivity 56 or 65%?

It means that only DCP has the sensitivity of **56**% and the combination of DCP with AFP has the sensitivity of **65**%

We are currently evaluating the accuracy of GPC3 for its adoption in clinical practice, and are preparing a manuscript associated with the usefulness of GPC3 as an early biomarker for recurrence.

Please eliminate, no relevance for readers or provide preliminary information about the results of this upcoming study.

We agree with this comment, so we deleted the following sentence on page 9, lines 21.

~~We are currently evaluating the accuracy of GPC3 for its adoption in clinical practice, and are preparing a manuscript associated with the usefulness of GPC3 as an early biomarker for recurrence.~~

The final conclusive sentences of the different biomarker all appear to be quite similar: it is a potential biomarker. E.g. "Thus, suPAR has potential as an early predictor to evaluate the risk of the development of HCC.", "Thus, SCCA-IgM IC may be a valuable serum marker for early HCC detection.", etc.
=> Please provide conclusions that are drawn appropriately supported by literature and try to compare the results to the AUC of the other discussed biomarkers.

We agree with this comment about SCCA-IgM IC, so we rephrased the following sentence on page 14, lines 23.

Although the AUC was lower than that of the other discussed biomarkers,

Because suPAR is different from other biomarkers in a valuable tool for the prediction of future HCC development in patients with benign liver disease, we think that the final conclusive sentences is not similar and appropriate.

P13: "inflammation, immune activation"
=> Please elaborate on the difference between these two terms.

Thank you for your comments.

We repeated similar phrase, so we rephrased the following sentence on page 15, lines 23.

the level of activation of the immune system

Incorporation of clinical variables like age and gender into models based on a combination of biomarkers for HCC detection further improve the predictive performance of these models. Please provide a literature overview of this strategy.

Thank you for your comments.
We added the strategy in the following sentence on page 20, lines 7.

Incorporation of clinical variables, such as age and sex, into models based on combinations of biomarkers could further enhance the predictive performance of the models for HCC detection.

"miR-130b showed a large AUC of **0.913**, with a sensitivity of 87.7% and a specificity of 81.4% for detecting HCC,"
=> Elaborate on this high AUC and clinical validation of this promising marker.

Thank you for your comments.
So we added the following sentence to elaborate on this high AUC and clinical validation of this promising marker on page 19, lines 9.

A panel of seven miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) has been shown to have high diagnostic accuracy in the early diagnosis of HBV-related HCC (BCLC stage 0 and A; AUC, 0.888)[101].
A few features, in addition to their expression profiles, make miRNAs particularly attractive as potential biomarkers. First, since many dysregulated miRNAs are highly stable and readily detected in serum and plasma in HCC patients, they may more generally have high AUCs in the detection of HCC as well as any other disease state.

"Thus, early diagnosis with a single biomarker is rather limited"
=> Please eliminate this sentence (repeated info)

We agree with this comment, so we deleted the following sentence on page 18, lines 21.

~~Thus, early diagnosis with a single biomarker is rather limited.~~

"For example, GPC3-targeted therapy, including with a peptide vaccine and antibody, showed some effect with good tolerance[54, 97]."
=> Please provide a more detailed description of the efficacy of GPC3-targetted therapy.

Thank you for your comments.
We added the result of our Phase I clinical trial using GPC3-derived peptide vaccines in the following sentence on page 20, lines 18.

In our Phase I clinical trial of GPC3-derived peptide vaccines, the disease control rate (partial response (PR) + stable disease (SD)) was 60.6% at two months after initiation of treatment. A median survival of 12.2 months was observed in patients exhibiting a high frequency of GPC3-specific cytotoxic T lymphocytes (CTLs) with no severe adverse events,

compared to 8.5 months in individuals with a low GPC3-specific CTL frequency (*P* = 0.033). GPC3 antibodies (GC33) had an SD of more than 26 weeks in 4 of 15 (16.7%) patients[103]. The median overall survival in the group with high expression of GPC3 (49.4 weeks) was greater than in the groups with low or no GPC3 expression (13.0 weeks).

Do you recommend the combination of OPN (highest sensitivity) and GPC3 (highest specificity)?

Thank you for your comments. As you mention, it could be a good combination.
However, there are many differences in those studies, such as trial designs, patient backgrounds and examination methods for biomarker, so further validations are needed.

In addition, even if these biomarkers are positive, therapeutic efficacy is still low in current treatment choices without imaging detection of the tumor. We also need the development of novel imaging modalities. Novel biomarkers may also help in explaining oncogenesis, ultimately leading to better treatment strategies.
=> Please rephrase this section and provide a strong conclusion and prespective.

We agree with this comment, so we added the following sentence on page 21, lines 8.

Finally, novel biomarkers may provide important clues to our understanding of oncogenesis, and ultimately lead to better treatment strategies. Simultaneous advancement in these many medical disciplines will hopefully initiate change in the poor prognosis of HCC patients.

Are the references according to journal guidelines?

We wrote the references according to journal guidelines with reference manager software (EndNote X7.3).

Table 2:

⇨ Please eliminate the name of the journals, the reference is sufficient.

We agree with this comment, so we deleted the name of the journals on Table 2.

⇨ Please add the discussed miRNAs to this table.

We agree with this comment, so we added the discussed miRNAs to table 2.

Reviewer 2

Please enter keyword “HCC”; “Biomarker”; “Early diagnosis” in Pubmed, you will find a lot of relevant updated atticles and reviews. I hope the author review through these papers, and add new associated research and progression in the submission to reorganize this paper. This paper is worth being improved. ?

We agree with this comment, so we added the updated articles.

Midkine (MDK)

MDK is a heparin-binding growth factor, initially identified as a retinoic acid responsive gene, which plays a critical role in cell growth, survival, migration, angiogenesis, and carcinogenesis[76]. In a study performed on patients newly diagnosed with HCC, MDK levels were found to be higher in cases of HCC than cirrhosis (0.625 *vs* 0.15 ng/mL; $P < 0.001$) or healthy controls (0.625 *vs* 0.125 ng/mL; $P < 0.001$)[77]. The AUC was at 0.941 (95% CI: 0.890–0.992), and for AFP at 0.671 (95% CI: 0.546–0.796) ($P < 0.001$). The sensitivity of MDK (0.387 ng/mL) to discriminate patients with early HCC (BCLC 0 and A) from those with cirrhosis was 90%, which was significantly higher than AFP (20 ng/mL) at 40%.

AXL

AXL is a receptor tyrosine kinase that has been implicated in the proliferation, survival and chemoresistance of many malignancies, including lung, breast, ovarian, colon and pancreatic cancers[78-82]. AXL is activated by the binding with growth arrest-specific protein 6 to the extracellular domain and undergoes proteolytic processing that results in the release of an 80 kDa soluble form that can be detected in serum[83]. Increased AXL expression has been identified as a poor prognostic factor for recurrence-free survival, as well as overall survival in colon and pancreatic cancer[80, 82]. The diagnostic value of AXL in early stage diagnosis of HCC (BCLC stage 0) was analyzed in a multicenter study[84]. The sensitivity of AXL (76.9%) was found to be much higher than that of AFP (38.5%), and AXL outperformed AFP (AUC, 0.848 *vs* 0.797 respectively) in detecting early stage HCC. Finally, AXL and AFP together reached an extraordinarily high AUC (0.936) in detecting early stage HCC, with sensitivity at 80.8% and specificity at 92.3%.

Thioredoxins (TRXs)

TRXs are thiol oxidoreductases that are ubiquitously expressed and involved in several biological processes such as, regulation of protein states, cellular apoptosis and proliferation, and protection against oxidative stress[85]. The expression of TRXs is increased in many neoplasms, and has been shown to correlate with prognosis, specifically in lung and colorectal carcinoma[86, 87]. Li *et al*[88] reported on the potential availability of a TRX for the detection of early stage HCC (well-differentiated, < 2 cm HCC). In this study, the sensitivity and specificity of TRX (74.9% and 87.5% respectively) were higher than for AFP (68.6% and 75.2% respectively). Furthermore, with an AUC of 0.854, TRX

outperformed AFP at an AUC of 0.720 in detecting early stage HCC. Again, when combined, TRX and AFP were more accurate in the detection of early stage HCC (AUC, 0.889; sensitivity, 81.3%; specificity, 93.4%).

Too much key words in the paper.

We agree with this comment, so we deleted the following key words.

~~annexin A2, nucleic acids,~~

the second/third leading cause of cancer-related deaths. I am confused. Second?Third?

Thank you for your comments. We corrected third to second on page 2, lines 3 and on page 3, lines 1.

second

In addition, the reference 1 and reference 4 is similar and one is in 2011, one is in 2013. Maybe 2013 is better. ?The first author in the references should be in bold. Right?

Thank you for your comments. We deleted the reference 1 and wrote the first author in bold in the references.

Please add associated references which are more updated and of high quality in this paper. The references already existed in thia paper need to be simplified.

reference 1 showed new case and death but reference 4 didn't show. And we couldn't find the later data than that of reference 1.

We agree with this comment, so we added associated references which are more updated and of high quality and deleted the following references.

Add references
[21], [49], [74]- [86], [89], [92], [99], [101]

Delete references
[1], [12], [15], [23], [26], [31], [34], [35], [37], [46], [55]

Please check the spelling and the layout in the whole paper carefully yourselves to avoid minor mistakes. Why a blank page in the end?

Thank you for your comments. We deleted a blank page.

Paragraph 2 in Page 3, "Unfortunately, even this is inadequate, and very few HCC

biomarkers have sufficiently high sensitivity for the detection of early stage HCC". I could not understand.

Thank you for your comments. As you pointed, there are some biomarkers which have high sensitivity for the detection of early stage HCC. So we rephrased the following sentence on page 5, lines 29.

Unfortunately, even this approach is inadequate, and very few HCC biomarkers demonstrate sufficient diagnostic performance for early stage HCC in clinical practice.

Paragraph 2 in Page 4, "AFP has been considered the most common and useful biomarker for HCC evaluation, because it was discovered in the serum of HCC patients in 1964[8]. " Here the use of because is weird.

Thank you for your comments. So we rephrased the following sentence on page 6, lines 23.

ever since

Reviewer 3

However, in the "Abstract" paragraph, the conclusions after the review should briefly mentioned.

We agree with this comment, so we added the following sentences on page 3, lines 10 and 18.

Advances in genomics and proteomics platforms and biomarker assay techniques over the last decade have resulted in the identification of numerous novel biomarkers and have improved the diagnosis of HCC. The most promising biomarkers, such as glypican-3, osteopontin, Golgi protein-73 and nucleic acids including microRNAs, are most likely to become clinically validated in the near future.

In this article, we provide an overview of the biomarkers that are currently used for the early diagnosis of HCC.

They defined that early-stage of HCC was BCLC stage 0 and A, the reported 5-year disease-free and overall survival rates of early HCC after treatment should be provided in the "Introduction" section of the text.

We agree with this comment, so we added the following title on page 5, lines 20.

In patients diagnosed with early stage HCC, such as Barcelona Clinic Liver Cancer (BCLC) stage 0 and A, the 5-year survival rate with surgical intervention was > 93%[4].