September 2, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: **12692** -review.doc).

Title: Management of hepatocellular carcinoma: Predictive value of immunohistochemical markers for postoperative survival

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Name of Journal: *World Journal of Hepatology*

ESPS Manuscript NO: **12692**

The manuscript has been improved according to the suggestions from You and Reviewers:

1. Format has been updated

2. Revision has been made according to the suggestions of the Reviewers. The changes in the manuscript are outlined in red.

3. Reviewer comments that need to clarify as below:

Reviewer#02929478

(1) ' Regarding the latter, in the section on HMGB1 DNA binding nuclear proteins, the authors could elaborate on how the process could be dramatically "sped up" when cytoplasmic localization of HMGB1 binds with RAGEs. '

Response: Following the Reviewer's suggestion, we have added the para below in regard to how the process could be dramatically "sped up" when cytoplasmic localization of HMGB1 binds with RAGEs (page 21, line 11):

' As for the "sped up" process, one study has deduced that the interaction between RAGE and HMGB1 activates mitogen-activated protein kinases (MAPKs), nuclear factor kappa B (NF-κB), and phosphoinositide 3-kinases/AKT signaling pathways to promote cellular proliferation and metastasis[163].'

163. Tang D, Kang R, Zeh HJ 3rd, Lotze MT. High-Mobility Group Box 1, Oxidative Stress, and Disease. Antioxid Redox Signal. 2011;**14**:1315-1335. [PMID: 20969478 DOI: 10.1089/ars.2010.3356]

(2)The second sentence of the section dealing with CK19 appears to have been misplaced (belonging in the CD133 discussion).

Response: Spot on! Thank you for your close attention!

'CD133 is currently recognized as a marker for LCSCs[181,183,195,196]. ' now reads 'CK19 is currently recognized as a marker for LCSCs[181,183,195,196]. '

(3) Finally, there are a small number of typographical errors.

Response: Again thank you for point it out and the following typographical errors have been corrected.

- Paragraph 3 under MMP9, Sentence 1: 'peritumour stromal cells' has been modified to' peritumoral stromal cells';
- Paragraph 2 under E-cadherin, Line 10: 'suggestlow' has been modified to'suggest low';
- Paragraph 2 under HMGB1, Line 12: 'clinicopathologic' has been modified to' clinicopathological'.

Reviewer#00069297

(1)"The contents would give significant information. "

Response: More details have been added to the contents in accordance with the more specific suggestions from Reviewer#02929478 and Reviewer#02860875.

(2)"The language need to be improved."

Response: Again, a number of modifications/improvements have been made in response to similar comments from Reviewer #02929478 and Reviewer #02860875.

Reviewer#00503849

This manuscript extensively reviewed the predictive value of almost all available immunohistochemical markers for postoperative survival in patients with hepatocellular

carcinoma. This manuscript can provide useful information to readers. I recommend publishing this manuscript.

Response: Many thanks.

Reviewer#02860875

Major

(1) 'I would think that the manuscript should be re-structured to make it more accessible. There is a discussion, within the conclusions, about what IHC features are important to become more widely clinically applicable. I think that this should be moved to the front of the article as this is fundamentally the most important aspect. What are the characteristics of the ideal IHC biomarker for HCC? Clearly it needs to be repeatable, with strong localized staining, valid across a number of patient groups and HCC subtypes, easily quantified and associated with clear clinical outcome measures.'

Response: We appreciate the Reviewer's concern and constructive input, and have come up with a different way of dealing with it - Paragraph 3 in Introduction has been re-written as follows:

Immunohistochemistry (IHC) is the most widely applied pathological technique in determining the expression status of tumor-associated proteins and in studying the prognostic and clinical relevance of biomarkers[7-9]. In spite of the paramount importance of IHC in determining the utility of a biomarker in clinical practice, the lack of universally accepted standardization guidelines has rendered the translation of promising biomarkers into clinical application. Having elaborated on nearly all the promising biomarkers so far in the main body of this review, we will discuss in Conclusion the various limitations and technical challenges that need to be addressed when validating via IHC a predictive biomarker for clinical endpoint. More specific to HCC, although many immunohistochemical markers have been reported to have a prognostic value for HCC patients, some of which are also validated as independent prognostic markers, so far, there has been no consensus on how these markers could add prognostic value to the clinical parameters. An ideal IHC biomarker for HCC needs to be repeatable, with strong localized staining, valid across a number of patient groups and HCC subtypes, easily quantifiable, and associated with clear clinical outcome measures. Based on our extensive review of relevant literature (summarized in Table 1), this review intends to find out why no immunohistochemical markers are applicable in clinical practice, and focuses on the most promising immunohistochemical markers among existing ones in predicting the postoperative survival of HCC patients.

(2) 'I would reduce the amount of discussion devoted to targets that are not realistic for implementation and concentrate on targets such as ki67 that are genuinely feasible for widespread clinical implementation.'

Response: As consented by the other reviewers, we have a slightly different view from this reviewer.

Indeed, there is some uncertainty regarding the methodological aspects of p53 immunohistochemical detection that may be responsible for the differences in frequency of p53 mutations and p53 protein levels, therefore, its clinical utilization is also hindered by the lack of standardized IHC methodology. Once IHC methodology is standardized, especially when a cut-off value of >10% is adopted, p53 immunopositive cells could be predictive of TP53 mutations in HCC. Hence, in our opinions, these discussions are necessary in the text.

(3) 'Further, we have to be clear about what we are using these markers for. If they are simply going to predict who is going to recur post-resection, is there any evidence for biomarker driven enhanced surveillance in altering that outcome? Or would they be better to guide resection versus percutaneous ablative therapy or directing the patient straight to consideration for transplantation.'

Response: In answer to this question, the following paragraph has been re-written for clarification purposes as the tenth paragraph of CONCLUSION AND PERSPECTIVES.

A number of studies have demonstrated that although single marker could provide useful information on the prediction of patients' survival and treatment outcomes, and could monitor efficacy of individualization of therapy, the heterogeneity of HCC tumors requires a combination of biomarkers in order to yield better clinical performance. In the foreseeable future it is likely that multiple markers need to be integrated into a prognostic signature to accurately predict outcomes. In fact, the HCC biomarkers in combination are increasingly becoming part of surveillance protocols in U.S. clinics[234].

234. Gish RG. Early Detection of hepatocellular carcinoma through surveillance using biomarkers. Gastroenterol Hepatol (N Y).2014;10:121-123.[PMID:24803876]

(4) 'Discussing a number of markers that are also mutated in HCC is slightly illogical. IHC will never tell us about the mutation status of these proteins.'

Response: The reviewer has a valid point here as IHC in itself couldn't tell us about the mutation status of these proteins. For emphasis purposes, the following paragraph has been added to the ninth paragraph of CONCLUSION AND PERSPECTIVES.

It is worthwhile to highlight that IHC in itself could never tell us about the mutation status of these proteins. That is to say, in order to better understand the relevance between immunohistochemical markers and clinical outcomes, standardized IHC should be combined with gene mutation analysis using PCR methods in the same patients.

Minor

(1) 'The review states in the abstract, core tip and introduction that hepatic resection is the 'most curative' treatment strategy for HCC. This is clearly not true, as liver transplantation has been demonstrated in multiple studies to have better long-term tumour related outcomes.'

Response: Thanks to the reviewer's comments, 'Hepatic resection(HR) is currently considered the best curative treatment modality for patients with HCC,' has been modified as below：

- In Abstract and Core Tip, it now reads: 'Hepatic Resection (HR) is generally considered to be one of the most effective therapy for HCC patients,'.
- In Introduction, it now reads: 'Hepatic Resection (HR) is a potentially curative and popular therapy for HCC patients'.

(2) 'In the introduction the authors state: 'and the major reason for the low postoperative survival rate is widespread intrahepatic metastasis or invasion[6]'. There are clearly 2 phases of tumour recurrence: early relating to intrahepatic metastases or occult synchronous HCC and late relating to metachronous HCC development. Therefore the above statement is not correct as stated.'

Response: We value the reviewer's correction and have made the following modification:

'and the major reason for the low postoperative survival rate of HCC is widespread intrahepatic metastasis or invasion[6].' now reads ' and the main reason for the low postoperative survival rate is either intrahepatic metastasis or metachronous multicentric HCC [6].'.

(3) 'In the section on TP53 the authors state: "role of p53 in tumor initiation as well as its malignant progression". I would restate to state that p53 is one of the most frequently mutated genes in HCC and that the loss of p53 is associated with a worse prognosis. I am not sure that wild-type p53 plays a role in tumour initiation.'

Response: This is a fair comment and we have made the following modification:

'p53 is probably one of the most widely investigated molecules in human HCC, and the role of p53 in tumor initiation as well as its malignant progression has been well characterized[10] ' now reads

'Alteration of p53 is one of the most frequent genetic change found in HCC, and the biological function of p53 in tumor initiation and progression has been well characterized[10].'

(4) 'You cannot state that on multivariate analysis p53 is not associated with outcome independent of tumoral phenotype and then 6 lines later state: "When combined with clinopathological parameters, there is an adverse impact of p53 expression on survival." The multivariate analysis shows that you can get the same information just from the clinicopathological data. '

Response: Thank you for pointing it out.

'When combined with clinopathological parameters, there is an adverse impact of p53 expression on survival.' has been modified into '…and it could have an indirect adverse impact on survival.' in the text.

(5) 'Fundamentally, IHC can tell you nothing about the mutation status of TP53. We know from sequencing studies that TP53 most often has missense rather than truncating mutatiions (Please see COSMIC database). IHC antibodies will always have difficulty in detecting proteins with a small number of missense AAs. Therefore the studies with high p53 expression by IHC reflect both high wild-type and mutant p53 which are clearly very different situations.'

Response: As suggested, the following content has been added to the main body as the ninth paragraph under p53 :

The detection of p53 expression by using IHC has another noteworthy problem. The p53 protein expression as detected by IHC does not always reflect the mutation status of TP53, with one cause being that not all mutations always result in stable protein formation, and another being that some tumors may also express wild-type p53. Nevertheless, in fact, lack of standardized IHC may be partly responsible for the inconsistencies in frequency of p53 mutations and p53 protein levels. TP53 most often has missense rather than truncating mutations, and IHC antibodies will always have difficulty in detecting proteins with a small number of missense AAs (amino acid substitution). Therefore the studies with high p53 expression by IHC may reflect both high wild-type and mutant p53. Given this, when determining p53 status in HCC, we should analyze it by standardized IHC in combination with p53 mutation analysis.

(6) 'When discussine E-Cadherin, you should discuss the significant literature describing loss of ECDH with the epithelial-mesenchymal transition.'

Response: As suggested, the following content has added to the main body as the fourth paragraph under E-Cadherin.

Decreased expression of E-cadherin has been found in all three types of epithelial–mesenchymal transition (EMT) and is thought to be the prototypical marker of EMT[99]. EMT has been shown to be a pivotal mechanism contributing to cancer invasion and metastasis, as epithelial cells lose their polarity and acquire the migratory properties of mesenchymal cells. The characteristic changes during EMT include the downregulation of epithelial markers such as E-cadherin and the upregulation of mesenchymal markers such as vimentin [100]. The EMT of HCC cells is thought to be a key event in intrahepatic dissemination and distal metastasis[101]. A recent study suggests that the loss of E-cadherin followed by the overexpression of vimentin may play a vital role in the invasive and metastatic phenotype and in the process of EMT, leading to unfavorable outcomes in patients with HCC[102].

99. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest. 2003 ;**112**:1776-1784. [PMID:14679171]

100. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelialmesenchymal transitions in development and disease. Cell. 2009;**139**:871–890. [PMID:19945376]

101. Zucchini-Pascal N, Peyre L, Rahmani R.Crosstalk between beta-catenin and snail in the induction of epithelial to mesenchymal transition in hepatocarcinoma: role of the ERK1/2 pathway. Int J Mol Sci. 2013;**14**:20768-20792. [PMID: 24135872 DOI: 10.3390/ijms141020768]

102. Mima K, Hayashi H, Kuroki H, Nakagawa S, Okabe H, Chikamoto A, Watanabe M, Beppu T, Baba H.Epithelial-mesenchymal transition expression profiles as a prognostic factor for disease-free survival in hepatocellular carcinoma: Clinical significance of transforming growth factor-β signaling. Oncol Lett. 2013 ;**5**:149-154. [PMID: 23255911]

(7) 'You have not mentioned any phospho-specific antibodies as potential biomarkers for HCC. These markers give some indication about pathway activation, rather than simply increased protein expression and therefore the potential for actionable targets. This would include phospho-S6 where several studies have demonstrated that evidence of mTOR pathway activation is associated with poorer outcome after resection (see for example PMID: 18929564).'

Response: Following the reviewer's advice, we have included mTOR pathway as a biomarker for HCC.

mTOR PATHWAY

Currently there is evidence suggesting that phospho-specific antibodies could serve as potential biomarkers for HCC. These markers provide useful reagents for analysis of signaling pathways in clinical samples, and therefore has the potential for actionable

targets[214]. So far, the molecular biology of hepatocarcinogenesis and HCC progression has been widely investigated. Many studies have indicated that signaling pathways dysregulated in HCC are important steps towards understanding HCC pathogenesis and developing new therapeutic approaches. Over recent years, several molecular pathways have been identified contributing to the molecular pathogenesis of HCC, among which the mammalian target of rapamycin (mTOR) signaling pathway has been identified to play a critical role in the pathogenesis of HCC[215]. And many studies have investigated the relationship between mTOR pathway and HCC prognosis.

mTOR pathway, an important regulator of multiple cellular functions including proliferation, differentiation, tumorigenesis, and apoptosis, is up-regulated in many cancers[216]. Deregulation of the mTOR signaling pathway has been reported in many malignancies, and some of the signaling molecules in this pathway could predict prognosis in different cancers. PI3K/AKT is considered a critical upstream mediator of the mTOR signaling pathway. Recent data from a genomic sequence of HCC samples identified mutations in PIK3CA, an upstream regulator of AKT, in 50% of patients with poor prognosis and survival length of < 3 years following partial liver resection, whereas only 10% of the HCC patients with a good prognosis had a mutation in PIK3CA[217]. Activation of AKT is a risk factor for early disease recurrence and poor prognosis in patients with HCC[218]. Activated AKT positively modulates mTOR function. mTOR is a key component of PI3K and AKT pathways that activate downstream kinases required for G1 to S phase transition[219]. mTOR acts by directly activating p70S6 kinase (p70S6K/S6K1) and inhibiting 4E binding protein 1 (4E-BP1)[220], both regulating the translation of important factors involved in cell proliferation (such as c-myc, cyclic D1 and pRb) and angiogenesis (such as HIF1-α)[221]. The p70S6 kinase and 4E-BP1 have shown to be independent predictors of prognosis in several types of solid tumors including liver[222-224]. Therefore, the expression of mTOR pathway could be used as prognostic indicator in HCC.

In addition, one study has indicated that JNK1 activation contributes to poorer HCC prognosis, and there is similarity in gene expression patterns between the HCC with abnormal mTOR signaling and JNK1 HCC[225], which further supports the assumption that HCCs with abnormal mTOR signaling are tumors of a highly aggressive nature and with poorer prognosis.

Recently, mTOR has emerged as an exciting target for cancer therapy including HCC. mTOR inhibitors have been tested successfully in clinical trials for their antineoplastic potency and good tolerability[226]. A second generation of mTOR pathway inhibitors has been utilized in preclinical HCC models[227] and the results suggest that mTOR inhibitors in HCC treatment could have a bright future.

Noticeably, although phospho-specific antibodies used in IHC are expected to detect phosphorylated proteins[228-230], some preanalytic variables (such as fixation technique and duration) may critically affect the signal[231], and in some cases these antibodies may also cross-react with nonphosphorylated proteins[232]. Therefore, it is of ultimate importance to standardize preanalytic variables and to employ a control in determining whether the staining pattern is specific.

214. Serrels A, Macpherson IR, Evans TR, Lee FY, Clark EA, Sansom OJ, Ashton GH, Frame MC, Brunton VG.Identification of potential biomarkers for measuring inhibition of Src kinase activity in colon cancer cells following treatment with dasatinib. Mol Cancer Ther. 2006;**5**:3014-3022. [PMID: 17148760]

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4. References and typesetting were corrected.

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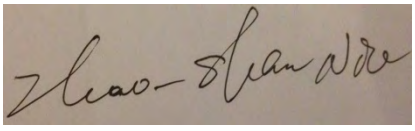
Sincerely yours,



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