

ANSWERING REVIEWERS

Feb 11, 2014

Dear Editor,



Please find enclosed the edited manuscript in Word format (file name: 8653-review.doc).

Title: Identification of biomarkers for hepatocellular carcinoma by semiquantitative immunocytochemistry

Authors: Hong Mu, Kaixuan Lin, Hong Zhao, Shu Xing, Cong Li, Fang Liu, Haizhen Lu, Ze Zhang, Yulin Sun, Xiyun Yan, Jianqiang Cai, Xiaohang Zhao

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 8653

We greatly appreciate the comments of the reviewers, and the manuscript has been improved according to their suggestions.

1. Format has been updated

We added the "COMMENTS" section according to the format for original articles.

2. Revisions have been made according to the suggestions of the reviewers.

Comments from Reviewer 1 (No. 02527378):

The paper is very well organized. The results obtained on cell lines and on blood samples are very interesting. Therefore the paper is acceptable in the present form.

Response: We appreciate the comments from the reviewer.

Comments from Reviewer 2 (No. 02860948):

Mu et al evaluated the expression of potential cellular biomarkers for hepatocellular carcinoma and demonstrated that ASGPR, GPC3, and CK may be valuable HCC surface biomarkers for circulating tumor cell detection. And they also suggested the EMT and stem cell-like related biomarkers as HCC recurrences and metastasis. There are a few issues which require to be addressed before considering publication.

(1) Please specify the title of the article including the methods and/or specific biomarkers.

Response: We thank the reviewer for suggesting to specify the title of the article. The title has been changed from the original one "A study evaluating the expressions of potential cellular biomarkers for hepatocellular carcinoma" to "Identification of biomarkers for hepatocellular carcinoma by semiquantitative immunocytochemistry".

(2) Authors only compared the HCC cell line or blood samples of HCC patients with normal control. However, HCCs usually came from the background of liver disease. Therefore, HCC samples have to be compared with liver disease such as cirrhosis or hepatitis sample.

Response: We thank the reviewer's constructive comments. We have added chronic HBV-infected patients for comparison. The fluorescence intensities of the three biomarkers (ASGPR, GPC3, CK) were significantly increased in HCC patients compared to those in chronic HBV-infected patients and healthy controls (see Figure 3 lower panel).

(3) Correlation with clinical data such as patients' survival or cancer recurrence will be helpful to determine the usability of biomarkers.

Response: We highly appreciate the reviewer's comments. Based on a multi-tissue array with clinical follow-up study data, we performed a survival analysis to assess the relationship between the expression of biomarkers and prognoses. The results indicated that patients with high ASGPR expression levels had poorer overall survival, and a similar trend was observed for GPC3 expression. Therefore, ASGPR and GPC3 expression may correlate with patient prognosis.

(4) Please state the reason for selection of CK, ASGPR, GPC3 et al as potential biomarkers for HCC.

Response: We thank the reviewer's comments. The reasons for selecting the biomarkers were added to the introduction section. We included HCC-related biomarkers, focusing on CK, GPC3, and ASGPR; stem cell-related biomarkers, focusing on EpCAM and CD133; and EMT-related biomarkers, focusing on vimentin. Cytokeratins are proteins in the intracytoplasmic cytoskeleton, and CK expression has been used as a biomarker in hepatoma histopathology. GPC3 attached to the cell surface is overexpressed in most HCC foci and undetectable in normal livers and benign liver diseases. ASGPR, as a membrane receptor, can specifically interact with the preS1 domain of HBV. EpCAM was reported as a HCC stem cell-like biomarker, and CD133 is a common stem cell biomarker. EpCAM- and CD133-positive cells can potentially undergo self-renewal and differentiation. Vimentin is a predominant mesenchymal marker in the EMT. Given that normal epithelial cells and hepatocytes in the circulation of healthy adults are rare, any cells containing the CK, GPC3, or ASGPR biomarkers are likely to be tumor cells. In addition, these biomarkers were selected based on our literature searching and proteomic analysis of normal liver tissues, HCC cells and tissues previously (eg. *Proteomics*. 2006, 6(19): 5260–5268; *J Proteome Res*. 2010, 9(1): 50-58; *J Proteome Res*. 2010, 9(1): 79-94).

Comments from Reviewer 3 (No. 02861016):

In this study, Mu et al address that a study evaluating the expressions of potential cellular biomarkers for hepatocellular carcinoma. The authors demonstrated that the immunofluorescence intensity to assess biomarkers' stainings for cells on coverslips and karyocyte dripping slides isolated and enriched from blood samples. ASGPR, GPC3 and CK may be valuable HCC biomarkers that could be used to detect CTCs in the blood. In addition, the expressions of EMT and stem cell-like related biomarkers could give us helpful informations on recurrences and metastases. COMMENTS Although the study is of potential interest and relevance, there is some space for improvement. In particular, as presented the study looks too descriptive in nature with little mechanistic insights for the observations made.

Response: We appreciate the reviewer's comments and have carefully improved the manuscript.

(1) The title is that a study evaluating the expressions of potential cellular biomarkers for

hepatocellular carcinoma. The authors should present more details to prove the new biomarkers for tumor recurrence and metastasis.

Response: We thank the reviewer's suggestion and did the same changes as responses to Reviewer 2's (1) and (3) questions to narrow the focus. Our results suggested that ASGPR, GPC3, and CK could be valuable HCC biomarkers for detecting CTCs in the blood. The CTCs identified by these three markers included tumor cells expressing EpCAM and vimentin, and the CTCs expressing stem cell-related and EMT-related biomarkers may display the potential for recurrence and metastasis identification. Also a survival analysis based on a multi-tissue array from clinical follow-up studies indicated that patients with high ASGPR expression levels had poorer overall survival, and a similar trend was observed for GPC3 expression. Therefore, ASGPR and GPC3 may correlate with patient prognosis.

(2) It is not good to present the figure legends in the result part and repeat results in figure legends and results. You must fit the author's guide.

Response: We thank the reviewer for noting the writing mistakes. We deleted the figure legends in the result section and moved them to the figure legend section.

(3) Are three biomarkers (ASGPR, GPC3 and CK) better than AFP in the serum to predictive tumor recurrences and metastases? What is the positive and negative predictive value of these three biomarkers for tumor recurrences and metastases?

Response: We thank the reviewer for the valuable comments. We compared the concordance ratios of the three biomarkers with that of AFP, and the ratios were higher than the concordance for serum AFP changes or AFP cellular expression previously reported. Additionally, the positive and negative predictive values of the three biomarkers were added to the manuscript. The concordance rates between a biomarker's expression and the pathological diagnosis were 90% for GPC3, 93.3% for ASGPR, and 63.3% for CK, whereas the concordance rate between AFP serum detection and pathological diagnosis was 46.7%. AFP was previously reported to be expressed in 52.3% of HCC patients (23/44) for the identification of CTCs. The positive and negative predictive values of these three biomarkers were 90% and 71.4% for GPC3, 93.1% and 75% for ASGPR, and 82.6% and 28.6% for CK, respectively.

(4) Several studies showed that EpCAM is a biomarker for HCC. What is difference between previous studies and your study.

Response: We thank the reviewer's comments. EpCAM in HCC tissues was reported to be a stem cell-like biomarker, and our innovation includes the followings: (1) the biomarker tests were performed on CTCs enriched from blood, a novel HCC sample type, in addition to the seven hepatoma cell lines; (2) EpCAM was used as a target to capture tumor cells from blood, but its expression was only approximately 30% on our karyocyte slides, which supports previous reports and suggests that several CTCs may not be captured and that our results supplement previous studies.

(5) There is minor language polishing.

Response: We thank the reviewer for noting the writing mistakes. The paper has been reviewed by a native English speaker from American Journal Experts as recommended by the journal.

Comments from Reviewer 4 (No. 02860538):

Mu and colleagues have provided a brief, original communication detailing an exploratory study of cytokine and receptor expression of potential biomarkers for hepatocellular carcinoma (HCC) prognosis. Circulating tumour cells were isolated from peripheral blood samples and purified prior to immunofluorescence staining. An additional in vitro study was performed investigating expression of the same biomarkers in hepatocyte cell lines. The prognosis for patients following diagnosis of HCC remains difficult to define. This is likely due to a number of factors including treatment modality and the specific aetiology of disease. This manuscript highlights a potential role for peripheral blood monitoring following diagnosis of HCC. There are several issues raised in the current manuscript which require addressing before publication can be considered. These are outlined below;

(1) The authors have used seven hepatocyte cell lines (Bel-7402, Bel-7404, SMMC-7221, HepG2, Hep3B, SK-Hep-I and Huh-7) as representative of HCC and the L02 cell line as a normal control. All cell lines are immortalised and thus the relevance of comparing in vitro results with patient samples for expression of HCC markers is not clear. When combined with the below suggestion, the omission of the in vivo results and discussion only of those results derived directly from examination of patient samples would provide more clinically relevant insight into the authors' hypotheses.

Response: We appreciate the reviewer's comments and agree with the opinions. Our manuscript reported a semiquantitative immunocytochemistry workflow to assess potential valuable biomarkers for hepatocellular carcinoma both in vitro (based on 7 immortalized cell lines) and in vivo (based on 30 patients' blood samples and 32 patients' tissues, see additional data in figure 5). In addition, we followed the "no harm principle" to perform the experiments, i.e., we tested the biomarkers on the cell coverslips first and then tested the more favorable biomarkers on karyocyte slides from patients' blood. Indeed, the results may provide more clinically relevant insight into the candidate markers.

(2) The authors state that peripheral blood was collected from 30 patients with HCC and 15 healthy controls. HCC may arise as a result of a number of different underlying causes (chronic viral infection, substance abuse, genetic predisposition etc.). While the authors state that 27 patients were HBV positive, more detail is needed to provide insight into the relevance of these biomarkers. With such small patient number a clear, well-defined cohort is required (eg. comparison of HCC markers in x number of mono-infected patients with HBV-related HCC, chronic HBV infection alone and uninfected controls). The inclusion of a subset of patients with chronic liver disease alone is of integral importance to this study as HCC is resultant of chronic liver damage. Biomarkers for HCC (such as α -fetoprotein) demonstrate perturbations from normal expression during chronic liver disease, the same factors should be investigated for any potential biomarker.

Response: We greatly appreciate the reviewer's comments and fully agree upon review's suggestion. We have added chronic HBV-infected patients to the comparison. The fluorescence intensities of the three biomarkers (ASGPR, GPC3, CK) were significantly increased in 30 HCC patients compared to 7 chronic HBV-infected patients and 15 healthy controls. The fluorescence intensities of the above three biomarkers were significantly increased in HCC patients compared to chronic HBV-infected patients and healthy controls although the increase of CK expression

was less pronounced than the other two biomarkers. The biomarkers could distinguish HCC from chronic liver disease and healthy controls. Furthermore, the test on the tissue array from 32 HCC patients provided the relationship between the biomarkers' expression levels and survival analysis for our manuscript. Our survival analysis from six and a half years' following-up indicated that ASGPR and GPC3 expression levels may correlate with patient prognosis. Of course, the sample size we tested is still limited, and a larger study with a clear, well-defined cohort will be required in the future.

(3) In line with the above, the authors state that a primary aim of their study is to aid in the prognosis of patients following diagnosis of HCC (ie. measuring patient survival, recurrence etc.). Yet this study does not provide any comparisons of clinical data with biomarker expression. These analyses would add significantly to the current study and should be performed prior to revision of the current manuscript.

Response: We thank the reviewer's comments. We have narrowed the topic and the title has been changed to "Identification of biomarkers for hepatocellular carcinoma by semiquantitative immunocytochemistry". Our manuscript reported a workflow to assess potential valuable biomarkers for hepatocellular carcinoma, including hepatoma-, stem cell-, and EMT-related biomarkers, through general immunofluorescence staining and fluorescence intensity comparisons. The results suggested that ASGPR, GPC3, and CK could be valuable HCC biomarkers for detecting CTCs in the blood. We added a correlation analysis between the biomarkers' expression levels and clinical data, and the results suggested that fluorescence intensity may not be affected by clinical pathological parameters. We also added the relationship between the biomarkers' expression levels and survival analysis to help clarify their clinical significance. Our survival analysis indicated that patients with high ASGPR expression levels had poorer overall survival, and a similar trend was observed for GPC3 expression. Therefore, ASGPR and GPC3 may correlate with patient prognosis.

(4) Additionally there are some minor issues which require addressing in revision of the current manuscript. - The manuscript should be re-structured to allow greater introduction to the individual biomarkers being tested in this study. There is no mention of the clinical relevance or indeed even the specific markers themselves until the discussion. This information should be included in the introduction. In this manner greater space can be devoted to discussion of the biological relevance of the results later in the manuscript. - There are a few spelling and grammatical errors which require addressing. In particular the authors are cautioned against the use of the word "expressions". No such word exists in the English language.

Response: We agree with the review's comment and have extended the introduction section to discuss and introduce the individual biomarkers being tested in this study; we also added information about their biological relevance to the discussion. Spelling and grammatical errors have been reviewed by a native English speaker from American Journal Experts as recommended by the journal.

3 Photo quality, references and typesetting were corrected

We have improved the qualities of the figures, such as merged original figure 1, 2 and 3 as a new figure (see figure 1); change figure 4 as a new figure 2; merged original figure 5 and 6 as a new one (see figure 3); merged original figure 7 and 8 as a new one (see figure 4); deleted the original

figure 9 (using words to state); and added the new data as figure 5 (see figure 5). The total number of references was adjusted to 54 as the format of *World Journal of Gastroenterology*.

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

Sincerely yours,

A handwritten signature in black ink, appearing to read 'Zhao' with a stylized flourish, followed by the date '2014-2-12' written below it.

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A handwritten signature in black ink, appearing to read 'HongMu' in a cursive style.

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