August 5, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 4670-review.doc).

Title: Association between RASSF1A promoter methylation and hepatocellular carcinoma: a meta–analysis

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Name of Journal: *World Journal of Gastroenterology*

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The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewers

(1) Reviewer No. 00068723

The methods of statistical analysis seem relevant. The authors proposed methylation of RASSF1A promoter is used as a diagnostic marker of HCC. Before the application to clinic, methylation of RASSF1A promoter should be discussed in the manuscript and understood by readers.

1) Why is it possible to analyze promoter methylation with fluid samples? Traditionally, promoter methylation was analyzed with surgically resected tumor samples (J Gastroenterol 48, 132-143, 2012). Is DNA isolated from the fluid samples?

Reply: Thank you for your comments. In this meta-analysis, we assessed the promoter methylation with fluid samples basing the fact that increasing abnormal cfDNA methylation patterns have been identified as novel non-invasive biomarkers for the diagnosis of human cancers (reference 6-10). The cfDNA in fluid might come from the release of extracellular nucleic acids in necrosis and apoptosis cells (reference 11). Several tumor-associated alterations including plasma/serum DNA methylation have been well demonstrated in liver cancer (reference 11) and such cfDNA is thought to be derived from poptosis and necrosis of cancer cells in tumor microenvironment (reference 31). We have modified this topic in the INTRODUCTION and DISSCUSSION part.

2) Biological significance of detection of methylation of RASSF1A promoter with serum samples should be discussed. Does that mean patient DNA already methylated? Or HCC tumor cells are circulating in the blood flow? Mohamed et al report that RASSF1A promoter is methylated in 10 % of control (reference 24). Does this mean false positive? If so, blood or fluid samples harbors false positive. The discussion about false positive would be desirable.

Reply: We totally agree with your opinion. Generally, methylated DNA can be detectable in serum samples due to the release of necrosis and apoptosis in HCC tumor cells (reference 11, 31). As you commented, the false positivity for RASSF1A methylation can be found in some cases and the following reasons might be: 1) RASSF1A gene promoter methylation might occur in controls including chronic liver disease and/or in preneoplastic (cirrhotic) liver to hepatocellular nodules to HCC (reference 24); 2) The qualitative tests including MSP used in some studies might have some difficulties in distinguishing true positive from false positive. Therefore the quantitative tests such as QMSP should be suggested to avoid the false positive. We have modified the issue in the DISCUSSION part.

3) Is methylation of RASSF1A promoter specific to HCC? If so, the methylation would be a marker of

HCC. If not, is the methylation a marker of various type of cancers? How did the author conclude methylation of RASSF1A promoter was a marker of HCC?

Reply: Thank you for your comments. The methylation of RASSF1A has been detected in a variety of cancers including HCC but it is not specific to HCC (reference 14). In this meta-analysis, we aimed to verify the feasibility of body fluid RASSF1A methylation in identifying HCC from high risk population rather than healthy population. So we selected patients with chronic liver diseases which were in high risk of developing HCC as controls. Under this condition, HCC was of greater possibility than other types of cancer. So methylation of RASSF1A is relatively specific to HCC and the detection was still meaningful in distinguishing HCC from benign liver diseases. We have modified the issue in the DISSCUSSION part.

4) Sensitivity and specificity vary depending on the publication. Some describe below 50 %. This phenomenon is common. How did the authors think about weight?

Reply: In this present study, we tried to analyze the heterogeneity source by meta-regression, but none of the three independent factors we considered were statistically significant to interpret the heterogeneity. We surmised that the limited number of included studies was the main reason that hampered the analysis of heterogeneity. As for the weight, the individual study results were all weighed for sample sizes and analyzed by using random-effect model according to the guidelines (reference 16).

(2) Reviewer No. 00070577

The authors showed that RASSF1A methylation in body fluids on HCC patients can improve HCC diagnostic accuracy using meta-analysis. The results are potential important, but I have some concerns about this paper.

1) Makers for HCC such as AFP levels are very different depending on the tumor differentiation and/or tumor sizes and/or number (stage). In this paper the authors did not show the data of tumor differentiation and/or tumor sizes and/or number (stage). Thus it is very difficult to say RASSF1A methylation is really useful compared to the AFP levels. I think this point is the weak point of this paper.

Reply: We totally agree with the opinion. Owing to the fact that we aimed to verify a marker which could be applied to non-invasive diagnosis, we only included studies using body fluids as samples, most of which lack surgically resected tumor samples as controls. So, information about tumor differentiation, tumor size, and number (stage) is vacant. We hope our future work may overcome the weak point.

2) The author showed the sensitivity of AFP, but did not show the specificity. The authors should show more data from literature. How was the sensitivity and specificity if the HCC was diagnosed by combination of AFP and PIVKA-II.

Reply: The sensitivity and specificity of AFP can be varied according to different cut-off value. In the study we referred (reference 2), the cutoff for normal AFP levels was 20 ng/mL on the basis of the EASL guidelines. However, the specificity was lacking because the survey included only HCC patients and focused on sensitivity. According to data from another article, the sensitivity is 0.422 and specificity is 0.949 when the cutoff value is 20 ng/mL (reference 29). The article also found that sensitivity and specificity was 0.944 and 0.756 respectively if the HCC was diagnosed by combination of AFP and PIVKA-II. We are excited about the findings. However, due to the fact that most of the studies included in our analysis failed to provide us with information about AFP level, we could hardly analyze the diagnostic accuracy of HCC by combination of AFP and RASSF1A. Still, more investigations should be done to identify this issue. We've added more data from literature and modified this part in DISSUSSION section.

3) Published papers usually mention the usefulness of a certain things, thus the paper having negative data often have not been published. Therefore it is possible the authors collected only papers that have only good data. The author should mention about this.

Reply: As we mentioned in the MATERIALS AND METHODS section, we performed a comprehensive literature search of articles published using multiple electronic databases and we rigorously included and excluded according to our criteria. What's more, we assessed our publication

bias by Deeks's test, and the results showed that no significant bias was found (P = 0.346). We mentioned the issue in the RESULTS section.

(3) Reviewer No. 00051753

The authors present a meta-analysis evaluating the performance characteristics of RASSF1A in assessing for hepatocellular carcinoma in at risk patients. As was pointed out, HCC is leading cause of morbidity and mortality, and current screening and surveillance tools leave much to be desired. The need for novel diagnostic biomarkers are needed, and the current paper presents a meta-analysis evaluating one of these potential tools. While the overall meta-analysis was clearly described and followed started algorithms for performing a meta-analysis study, my main concerns reside in the heterogeneity of the studies included. Given that this is a novel biomarker, extreme care is needed not to conclude a false association when one does not exist. The studies that were included in the current meta-analysis had sensitivities ranging from 0.27 - 0.94 and specificities ranging from 0.38 - 0.95. This represents huge variation, and extreme caution is needed when interreting pooled values that incorporate such wide variations from studies that have relatively small sample sizes. Furthermore, the "control groups" of the different studies included are not entirely comparable. The different cohorts include HBV, HCV, cirrhosis, and presumably non-cirrhotic, although this is not clear in the study. It is possible that RASSF1A may perform differently in the setting of HBV, HCV, cirrhosis, and this should be addressed by the authors. Despite these major concerns, I think the idea of this paper is novel and important as we continue to evaluate novel potential biomarkers for the early diagnosis of HCC.

Reply: We greatly appreciate your constructive comments and suggestions for our paper. The two issues you mentioned are obviously limitations of our work and we discussed them in the DISSCUSSION section. We hope our future work could solve the problems and be of better quality.

Minor comments:

1) In the study characteristics section of the results, it currently indicates that Taiwan is a region that is part of China. While this may reflect the opinion/perspective of the authors, the World J Gastroenterology targets an international audience, where Taiwan is recognized as a separate country. Thus, this should be corrected.

Reply: We've made corresponding modification in the revised edition. Thank you for your comments and we totally agree with you that the spirit of science is out of politics. We understand that Taiwan and Hong Kong are special areas and we have modified this issue carefully in the revised table 1.

2) Review of the manuscript for language assessment is needed, as there are minor English language stylistic/grammatical items that need to be corrected prior to publication

Reply: According to your constructive comments, the English writing of this revised manuscript has been given proof-reading by Jing-Yun Ma Office for SCI Biomedical Editing and Publishing(Certificate verification code: 2013-08-265). In the certificate verification, they declared that the revised manuscript has reached grade A as defined by WJG language evaluation.

3 References and typesetting were corrected

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

Sincerely yours,

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