Format for ANSWERING REVIEWERS

November 02, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 13834-review.doc).

Title: Methylation of *IRAK3* is a novel prognostic marker in hepatocellular carcinoma

Author: Chih-Chi Kuo, Yu-Lueng Shih, Her-Young Su, Ming-De Yan, Chung-Bao Hsieh, Chin-Yu Liu, Wei-Ting Huang, Mu-Hsien Yu, Ya-Wen Lin

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 13834

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 reviewer

(1) Reviewed by 00069618

Due to the lack of reliable biological markers for prognosis of HCC, the role of IRAK3 is promising. However the authors should better describe how they think to translate this research in clinical practice. Minor point: in the first line of abstrct correct heptocellular to Hepatocellular

Response:

We appreciate your positive comments and suggestions. The methylation biomarker is relatively stable in tissue samples and body fluids, suggesting that it is a promising tool to identify or monitor diseases. The methylation biomarker can also reflect the exposures of the environment, determine the onset or course of diseases, and examine a patient's response to therapy. These examples further indicate that it has proven to be a promising strategy to diagnose or predict diseases. In this study, we showed that *IRAK3* methylation was positively associated with tumor stage and poor 3-year disease-free survival of HCC patients. These results suggested that we could detect *IRAK3* methylation in tissue sections to predict the survival of patients. It would help doctors to know which patients should be followed up intensively. We have incorporated this statement into the sections of discussion in our revised manuscript (page 15). In addition, we are sorry for the typing error. The type error in the abstract has been corrected in our revised manuscript (page 3).

(2) Reviewed by 00503404

The paper is investigating the importance of the methylation of the IRAK3 during the progression of HCC and was associated with tumor stage and poor prognosis of patients Comments; 1. the technique and cohort size was appropriate. 2. A question would be if authors could further digest the stage/causative agent association, in other words was there the same trend in different HCCs, for the stage IRAK association? Please analyse the HCV-HBV-negative patients separately. 3. Authors could/should calculate HRs for the time dependent models by using COX regression

1. the technique and cohort size was appropriate

Response:

We are appreciated for your positive comments.

2. A question would be if authors could further digest the stage/causative agent association, in other words was there the same trend in different HCCs, for the stage IRAK association? Please analyse the HCV-HBV-negative patients separately.

Response:
We appreciate your comments and suggestions. Interestingly, while dividing the 57 patients by hepatitis, we found that there was a similar trend toward 3-year disease-free survival in different HCC cases (supplemental data for review Figure S1). But only in patients without hepatitis was *IRAK3* methylation significantly associated with tumor stage (p=0.03) and poor 3-year disease-free survival (p=0.03, n=20). However, confirmation of this association requires further investigation in large-scale studies of clinical samples.

3. Authors could/should calculate HRs for the time dependent models by using COX regression

Response:
We appreciate your comments and suggestions. By Log-Rank test, our present result showed that *IRAK3* methylation has a trend toward poor 3-year disease-free survival (p=0.04, n=57). Cox regression analysis (supplemental data for review Table S1) showed that the risk for death of patients with *IRAK3* methylation is 6.5 times greater than that of patients without methylation. Even though there is a borderline trend of death in patients with *IRAK3* methylation (HR=6.5, 95% CI=0.84-51.2), the Log-Rank test showed *IRAK*3 methylation has a significant trend toward poor 3-year disease-free survival (p=0.04). These results suggest that *IRAK3* methylation has a positive trend toward poor 3-year disease-free survival. However, it needs to be further confirmed in large-scale studies of clinical samples.

(3) Reviewed by 01943107

In this study the authors demonstrated that IRAK3 and GLOXD1 gene expression was down-regulated in HCC cell lines and that it was partially restored after treatment with 5DAC. Importantly, they also found that IRAK3 methylation was statistically associated with tumor stage and with a trend of poor 3-year disease-free survival in HCC samples. Data are very interesting; however there are some critical points. 1. HCC cell lines exeperiments: in my opinion the addition of ChiP methylation assay could reinforce data obtained in cells lines. Moreover, more deatails than Refs 16 should be provided about methods.

Response:
We appreciate your comments and suggestions. In cell lines experiments (Figure 1), we showed that *IRAK3* and *GLOXD1* were frequently down-regulated in HCC cells via the promoter methyation. This indicated that *IRAK3* and *GLOXD1* expression might be regulated by DNA methylation. Therefore, we further focus on examining gene methylation in tissue samples by quantitative analysis (including pyrosequencing and Q-MSP) and their clinical applications. *IRAK3* and *GLOXD1* were selected from our previous study using Infinium HumanMethylation 27K. In general, methylation level could be confirmed by bisulfite sequencing, pyrosequencing, and QMSP. We used RT-PCR, pyrosequencing, and Q-MSP to prove that *IRAK3* and *GLOXD1* were frequently down-regulated in HCC cells via the promoter methylation. We would not use ChiP methylation assay to prove this part. The methods about Infinium HumanMethylation 27K and 450K BeadChip have been described in Refs 16 and our previous study (Refs 18). We did not describe the methods in detail to avoid redundant publication and self-plagiarism.

2. HCC liver samples experiments: data on the Real-Time PCR expression of IRAK3 and GLOXD1 in HCC and non-HCC samples should be reported and correlated with methylation status and clinical features by adequate statistical analysis.

Response:

We appreciate your comments and suggestions. In the cell lines' experiments (Figure 1A), we showed that *IRAK3* and *GLOXD1* were expressed in normal control and THLE-3 cells and down-regulated in HCC cell lines as inversely correlated with methylation status. Furthermore, our preliminary data showed that down-regulation of *GLOXD1* was frequent in HCC tissues as compared with nontumor tissues (supplemental data for review Figure S2A, n=14). Down-regulation of *GLOXD*1 was correlated with methylation status (supplemental data for review Figure S2B). Expression of *IRAK3* was low in nontumor tissues and undetectable in HCC tissues. However, there is only a marginal negative trend between *IRAK3* methylation and mRNA expression in HCC tissues due to limited samples. These results implied that the down-regulation of *IRAK3* and *GLOXD1* in HCC may be partly through the promoter hypermethylation. Confirmation of this data requires further investigation in large-scale studies of clinical samples.

3. Methods section should be presented as in order of results.

Response:

We are appreciated for your comments and suggestions. We have modified the methods section as in order of results (cell line experiments, patients, and quantitative methylation analysis).

4. Editing English revision is required.

Response:

We are appreciated for your comments and suggestions. We have made the editing service provided by professional English language editing company (American Journal Experts: http://www.aje.com).

5. Check abbreviations and reference format as requested by the Journal.

Response:

We are appreciated for your comments and suggestions. We have checked the format as requested in our revised manuscript.

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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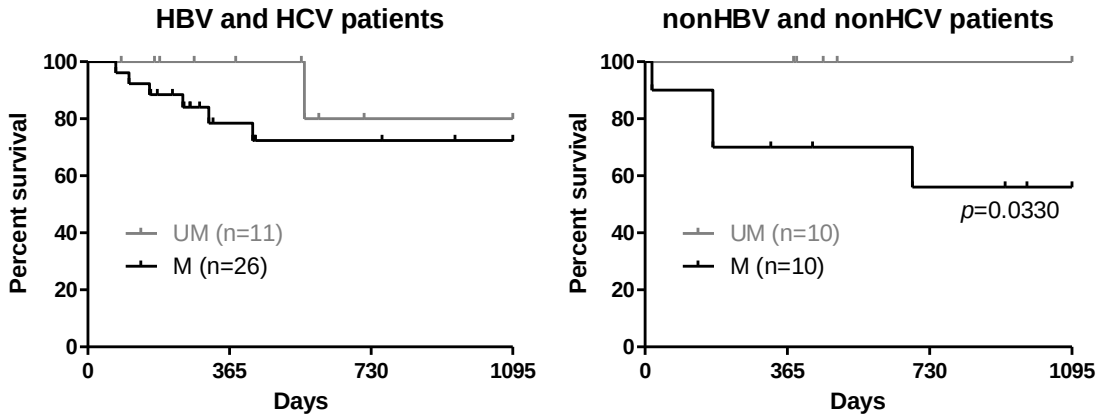

Figure S1 Correlation analyses between *IRAK3* methylation and the survival of HCC patients with or without hepatitis. Survival analysis was analyzed by Kaplan–Meier curves. The plots were made according to the patients with (n=37) or without hepatitis (n=20) and 3–year-disease-free survival in a total of 57 HCC patients, respectively (Log-Rank test, UM, unmethylated cases vs M, methylated cases).

Table S1. Cox regression analysis for survival of 57 HCC patients

Event	3-year-disease free survival
Variable	HR (95% CI)
IRAK3	
Unmethylation	1.0 (reference)
Methylation	6.5 (0.84-51.2)

HR, hazard ratio; CI, confidence interval.

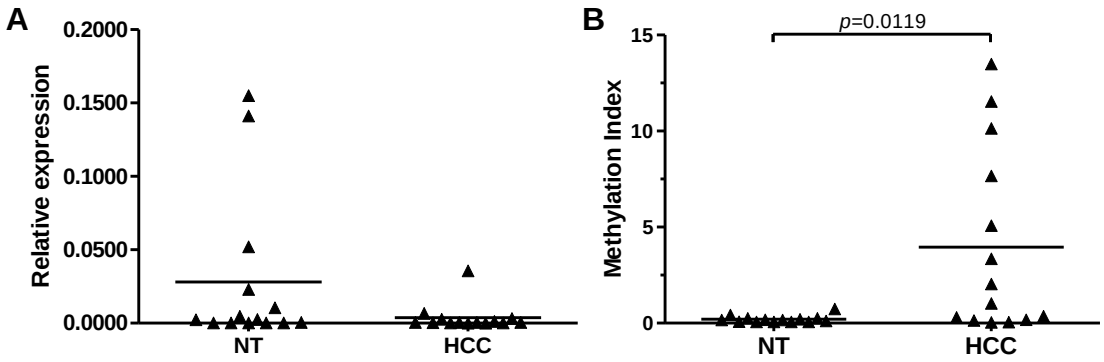

Figure S2 Expression analysis and methylation analysis of *GLOXD1* in nontumor and HCC tissues. (A) *GLOXD1* expression was analyzed by Q-PCR in 14 pairs of HCC and adjacent nontumor (NT) tissues. Relative gene expression was determined based on the quantification cycle value (Cq value) of the gene of interest (*GLOXD1*) and of the internal reference gene (*GAPDH*), using the formula: $2^{[-\Delta Cq]}$. The ΔCq value of *GLOXD1* for each sample was used the formula: Cq of *GLOXD1* - Cq of *GAPDH*. The black lines indicate the mean of relative expression level for analyzed samples. (B) *GLOXD1* methylation was analyzed by Q-MSP in 14 pairs of HCC and adjacent nontumor (NT) tissues. The black lines indicate the mean methylation index. (p=0.0119, paired t-test).