Replying to the reviewer's' comments:

October 7, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 4969-review.doc).

Title: ALK gene copy number gain and its clinical significance in hepatocellular carcinoma

Author: Shou-Wei Jia, Sha Fu, Fang Wang, Qiong Shao, Hong-Bing Huang, Jian-Yong Shao

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 4969

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

Reviewer number: 1

(1) The INTRODUCTION is unstructured and extends for more than 470 words.

Reply: We thanks to the reviewers for this suggestion. We have re-written the INTRODUCTION section and words were reduced to 442 words.

(2) Discussion of the results is too brief and should be elaborated more.

Reply: Thank you for this excellent suggestion. We have re-written the discussion section according to your together with the other two reviewers' comments. We have added the discussion of the results in the revised manuscript.

Reviewer number: 2

Major Compulsory Revisions

(1) This study suggests that the ALK/CNG positivity has clinical implication in advanced HCC patients. Therefore, the gene copy number gain of ALK is the phenomenon that develops along with the progression of the tumors but not early event of the tumor. This should be clearly notes in the discussion.

Reply: Thank you for this excellent suggestion. We have re-written the discussion section according to your comment. We have added the discussion of ALK/CNG in advanced HCC patients in the revised manuscript according to the review's comment. Details can be seen on Page 13, Line 2 in the revised manuscript.

(2) Did the authors examine the amplification of genes other than ALK that have been reported to be of clinical importance?

Reply: We didn't examine the amplification of genes other than ALK.

(3) The authors investigated the ALK gene alteration and discussed on it. However, they did not mention the gene expression of ALK. There appeared the following report. Shao CK, Su ZL, Feng ZY, Rao HL, Tang LY. Significance of ALK gene expression in neoplasms and normal tissues. (Article in Chinese) Ai Zheng. 2002 Jan;21(1):58-62.

Reply: Thank you for your helpful suggestion. We have read the paper which was published in Ai Zheng. 2002 Jan;21(1):58-62. We considered that ALK gene copy number might influence on the levels of ALK protein expression. However, there are too many factors that will affect protein expression in different signaling pathways. According to the references reported, there are two novel monoclonal antibodies recognizing the ALK protein. The mouse monoclonal antibody ALK1 was raised against a peptide encoded by amino acids 1359–1460 of the human ALK protein. The rabbit monoclonal antibodies D5F3 and D9E4 were raised against a peptide derived from the

c-terminal portion of the human ALK downstream of the kinase domain and preserved in NPM-ALK, EML4-ALK, and all other known pathologic ALK fusions. Since we are not sure which kinds of ALK subtypes altered in HCC, so we did not stain the ALK expression level to correlate to ALK gene copy number in HCC. In the future, we will investigate ALK expression level and correlate it to ALK gene copy number in the future research. Furthermore, we discussed the ALK protein expression level and its clinical significance reported by Shao CK et al. and cited this paper in the revised manuscript.

(4) The first paragraph in the discussion seems to be a review without referring the present study results.

Reply: Thank you for helpful suggestion. We re-wrote the discussion section of the first paragraph and added the present study results according to your comment. Details can be seen on Page 12 Line 10 in the revised manuscript.

(5) The design of this study seems to be retrospective study. This should be clearly described in the manuscript.

Reply: Thank you for helpful suggestion. In order to clear state this is a retrospective study, we have added “retrospective study” information in the introduction section, last paragraph, the first sentence on Page 5, Line 8; as well as added “retrospectively” in the section of the results, on page 9, Line 3. according to the review’s comment.

Minor Essential Revisions

(1) In the Results section, “The cancer TNM stage was defined according to the 1997 American Joint Committee on Cancer (AJCC) staging system. Cancer histopathological classification was

defined according to World Health Organization classification criteria" should be moves to the Materials and Methods section.

Reply: Thank you for your suggestion. We have moved the sentences to the Materials and Methods section in the revised manuscript.

(2) The reviewer can't understand how the values derived from: "The OS rates showed a marginal statistically significant difference between the ALK/CNG positive and ALK/CNG negative HCC patients (21.4% vs. 32.4%; P = 0.089) (Figure 2A) in page 8".

Reply: Thank you for your suggestion. We have re-written the sentences to clearly state the clinical significance as "The 3-year OS rates did not differ significantly between subgroups of ALK/CNG-positive and ALK/CNG-negative HCC patients (21.4% vs. 32.4%; P = 0.089) (Figure 2A)." in the revised manuscript. Details can be seen on Page 9, Line 23 in the revised manuscript.

(3) "In addition, HCC patients with ALK/CNG had a significantly poorer prognosis than other patients (3.6% vs. 28.5%; P = 0.048)" in the abstract. The values presented in % need more explanation, such as survival rate at 3 years. In page 9, again, the values presented should be reconsidered: "In advanced stage (stages III-IV), the 3-year OS and PFS rates for ALK/CNG positive patients were 15.4% and 10.3%, respectively, which were significantly poorer than that of the ALK/CNG negative HCC patients (0% vs. 18.8%; P = 0.054 and 0% vs. 12.5%; P = 0.007, respectively) (Figure 3A and 3B)"

Reply: Thank you for your suggestion. We have re-written the sentences to clearly state the clinical significance in the revised manuscript. Details can be seen on Page 10 in the revised manuscript.

(4) The following sentence appeared in the abstract should be revised. “Patients with progression-free-survival in the advanced stage (stages III-IV) and overall survival in Grade III had statistically less ALK/CNG than early stage/Grade II patients (0% vs. 12.5%; P = 0.007 and 13.3% vs. 28.9%; P = 0.023, respectively)”

Reply: Thank you for your suggestion. We have re-written the sentences in the abstract section according to you comment in the revised manuscript. Details can be seen on Page 2, Line 14 in the revised manuscript.

(5) In page 11, it is not clear what kind of failure that the authors intended to mention: “HCC has a high failure rate …”.

Reply: Thank you for your question. We have re-written this sentence as “HCC has a high therapeutic failure rate and a low median survival rate because of the aggressive nature of the disease” in the revised manuscript. Details can be seen on Page 12 Line 2 in the revised manuscript.

(6) In the supplementary Table 1, the values should be better presented in the order of ALK/CNG, such as ALK/CNG>6, ALK/CNG>5, ALK/CNG>4, and ALK/CNG>3.

Reply: Thank you for your suggestion. We have reformed the supplementary Table 1 to present the ALK/CNG in the order of values in the revised manuscript. Details can be seen in the revised supplemental Table 1.

Reviewer number: 3

(1) The PFS rates showed a statistically significant difference between ALK/CNG positive and ALK/CNG negative HCC patients. Recurrent HCC was categorized into two groups prior to the study, as intrahepatic metastasis recurrence or multicentric recurrence. Background liver affects postoperative multicentric recurrence. However, in the recurrence free survival analysis, there were no mention of background liver function, such as Child-Pugh score, platelet count and state of viral hepatitis. You should re-analysis the survival, along with these factors.

Reply: Thank you for your excellent suggestions. We have analyzed the survival along with the Child-Pugh score, postoperative platelet count and state of hepatitis B viral in the revised manuscript according to your comment. Details of these data are shown in Figure 5 and Supplementary Figure 3-4 in the revised manuscript.

(2) Why ALK gene copy number affected the survival of HCC patients, in spite of no characteristic difference such as stage, pathological grade, AFP level, and recurrence rate between 2 groups? How you presume about the mechanism of ALK gene number that affect prognosis? If ALK gene affects cancer cells proliferation, you should analyze the relation between some of tumor volume of HCC patients and ALK gene status. If ALK gene affects tumor invasiveness, you should evaluate about vascular invasion or growth patterns and ALK gene status.

Reply: The correlation between ALK status and clinicopathologic variables was assessed by $\chi 2$ test or Fisher's exact test. If P-value was considered statistically significant, it is important to select targeted therapy strategies according to the specific clinicopathologic features of the patient tumors. Survival analysis was estimated using the Kaplan-Meier approach with a log-rank test. Univariate and multivariate analyses of clinical variables were performed using the Cox proportional hazards regression model. The probabilities of OS and PFS were showed a statistically significant difference,

the results suggest that ALK gene copy number may affected the survival of HCC patients. In our study, the PFS rates showed a statistically significant difference between ALK/CNG positive and ALK/CNG negative HCC patients, and we suggested that HCC patients with ALK/CNG affected the survival of HCC patients.

We have speculated the mechanism of ALK/CNG that affect prognosis in the section of the discussion. Details can be seen on Page14, Line 2 in the revised manuscript.

(3) Regarding the study design. In this study, the authors used ≥ 4 copies per cell in ≥40 of 100 cells analyzed as a cut-off for ALK/CNG positivity based on the overall consistency of survival data, because the criteria for ALK/CNG has not been established. But I think it may lack in persuasiveness if you want to newly establish the criteria. So, you should set a cut-off value according to calculated ROC curve. If it could not do it, please state the reason not to be able.

Reply: Thank you for your excellent suggestions. We have re-analyzed the data and using ROC curve for cut-off calculating ALK/CNG positivity in the revised manuscript according to you comment. Details can be seen in Supplementary Figure 1, as well as in text on Page 7, Lines 14 in the revised manuscript.

(4) In this study, the primary endpoint was overall survival (OS). However, there was no significant difference between 2 groups in OS. So, I think the final conclusion sentence is too strong and not entirely supported by this paper.

Reply: Thank you for your good suggestion. We modified the sentence as “The endpoints were overall survival (OS) and progression-free-survival (PFS)” on Page 8, Line 4; Also in final conclusion, we have re-written the sentences to reduce stronger sentence in the revised manuscript

according to your comment. Details of this can be seen on Page 14, Line 10 in the revised manuscript.

(5) You should cite a reference about "ALK belongs to the insulin receptor superfamily of tyrosine kinase receptors –", at page 11, line 12 of Discussion section.

Reply: We added the reference about this sentence in the revised manuscript according to your comment. Details of this can be seen on Page 12, Line 14 in the revised manuscript.

3 References and typesetting were corrected

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

Sincerely yours,

Jian-Yong Shao, MD, PhD

Department of Molecular Diagnostics,

Sun Yat-sen University Cancer Center.

Guangzhou, 21 Qing Cai Gang Road,

H-1083- China,

Fax: +86-020-87345599

E-mail: shaojy@sysucc.org.cn.