Format for ANSWERING REVIEWERS

May 24, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 10818-review.doc).

Title: Circulating Tumor Cells and Cancer Stem Cells in HCV -Associated Liver Disease

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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 reviewer

(1) **02861277**

(2) **02861340**

(3) **02861333**

General Comments

1- The manuscript has been structured according to journal style and all general comments including: front page, title, author …etc have been done as requested

2- All the changes done have been highlighted in red in the text.

Reviewer NO.: 02861277

1) **The authors reported no significant differences concerning CK19 expression between HCC and CH groups (page 10), while considering the cut-off they become significant (please clarify).**

Authors Response: As a first step, we compared the median of CD45-/CK+ cells for the three studied groups and the results showed that there was a significant difference between the control subjects and the two other groups (CH/HCC). Then we thought to assess whether there is a cutoff for CK19 that can help to differentiate between the two patient groups (CH/HCC) since the aim is to define prognostic and predictive biomarkers for HCV-associated HCC. So, we find that, at the specified cutoff (73) it could be possible to differentiate between the two groups. As you can see it is very much similar to breast cancer, where some researcher consider ≥4 circulating tumor cells (CTC) a positive result while others consider ≥5 CTC positive and still others consider ≥5 CTC positive.

2) **Table 3: standard deviation is higher than the respective values, how is possible? (Please clarify)**

Authors Response: This is because of the outliers (some data points within a group is varied)

3) **Figure 2: representative dot plots for all groups should be shown.**

Authors Response: This has been done and added as a separate figure (Figure 3)

4) **Real time PCR: Has RNA extraction been performed from all cells stratified after centrifugation, included leukocytes? (Please clarify).**

Authors Response: Yes the real time PCR was performed on the separated mononuclear cells which include leucocytes in order to determine the approximate amount of CTC in the sample via determination of the expression level CK as an epithelial cell marker. This was done to confirm and support the data of the FCM. The presence of leukocytes will not add bias to the data since 1) all cases were managed the same way and 2) the WBCs are negative for CK.

5) **Minor aspects: A legend for acronyms should always report.**

Authors Response: A list of appreciations was added as requested by the reviewer

Reviewer NO: 02861340

1) **How many patients are HCV-based and HBV-based HCC? How about the comparison of these markers in HCV- and HBV-based HCC?**

Author response: All cases were HCV positive and none was HBV positive as mentioned both in the Abstract and in the Methods sections. Abstract: we assessed the role of CTCs and CSCs in chronic HCV infected Egyptian patients. Methods: We also included 30 post-HCV-CH patients who were diagnosed by clinical examination, abdominal ultrasound, laboratory investigations and liver biopsy as well as 33 healthy volunteers matched for age and sex with patients as a control group. They all had normal values of serum alanine aminotransferase (ALT) and were sero-negative for hepatitis B surface markers (HBs Ag, HBeAg and HBc-Ab) and HBV antibodies. Another set of 50 pathologically confirmed HCC fresh tissue samples (26 males and 24 females) was also included as a confirmatory set (CS) to validate the data obtained from the OS. All cases were HCV positive/ HBV negative, newly diagnosed cases. None of the HCC patients received prior chemotherapy.

2) **Why did the authors focus on CK19, telomerase, MAGE1, MAGE3, CD90 in this study? It would be nice to more clearly describe the designing of the study.**

Authors Response: CK is a well known epithelial marker that is used in almost all research work for detection of CTCs either by PCR, FCM or the cell search system. The other markers (telomerase, MAGE1, MAGE3, CD90) were chosen because many previous studies in literature have shown a correlation between the expression levels of

these markers in blood and/or tissues and both CH /HCC either as predictive, prognostic or markers for early detection. This has been well illustrated in the " methods" section- Page 8.

3) **The authors examined the expression of these markers only in NC, CH, and HCC. Do the markers not express in other tissue cancers or other pathogenic conditions in the liver?**

Authors Response: We did not look for this in our study because our aim was to assess the expression levels of these markers in chronic HCV- infected patients and HCC. However, we know from the literature that they could be present in other cancers however, MAGE1/MAGE3 were linked to HCC more than to other types of cancer. Similarly, CD90 and CD133 have been mentioned as cancer stem cells related to HCC and other cancers though more common in HCC. So, what we are interested in this study is to compare between the expression levels of these markers in the cascade of HCV-associated hepatocarcinogenesis, especially in the Egyptian population because 1) this has not been addressed in this population before, and 2) because of the unique genotype (genotype 4) which is responsible for almost more than 90% of the population. We want to see whether any/or all of them could be used for early detection, prediction or assessment of the prognosis in those patients.

4) What's the advantages of the markers clarified in this study over those currently used in clinical, especially the AFP?

Authors Response: As mentioned in the introduction and discussion sections "till now the choice of therapy and the prognosis depend largely on the severity of liver function, radiology image, and AFP. However these parameters proved to be insufficient for predicting patients' outcome and therefore, individually-based biological markers seem to be highly required.

According to our results 1) enumeration of CTCs by FCM using, CK and CD90, has high sensitivity and specificity to improve prognostic accuracy, monitoring therapeutic outcomes, and predection of HCC in HCV infected patients. 2) Aberrant expression of HCC specific and CSC markers (CD90, MAGE3, telomerase, CD133 and CK19) contributes to poor prognosis in HCC patients and can help in better management of patients. However, the number of the studied cases is too small to reach to a strong conclusion and therefore, a larger study is running now in Egypt to confirm this data and putting the results in the clinical setting (CONCLUSIONS PAGE 16 & 17).

Reviewer NO. 02861333:

1- In the abstract, "cancer stem cell (CSC) were recently used", should be "cancer stem cells (CSCs) were recently used".

Authors Response: This has been corrected all through the manuscript.

2- Why the number of CD133+ cells was lower in HCC than CH group?

Authors Response: This is already discussed in the "Discussion" section, page 14: CD133+ cells were significantly higher in CH patients. One possible explanation for this finding is that CD133+ cells also represent a subset of (normal) stem cells, which is released from the bone marrow into the circulation during the early inflammatory stage of HCV-associated liver disease in an attempt to repair the hepatic damage, compensate for the cell loss and prevent or remove the fibrosis induced by the virus. However, with failure to clear viral infection and/or repair the damage, the CD133+ cells, having the plasticity and the ability of unlimited proliferation, will set the stage for the development of HCC on top of the chronic inflamed and possibly cirrhotic liver.

Kindly see our publication in the role of stem cell therapy in the treatment of the end stage

liver diseases (Salama et al., 2010, 2012,2014).

3- Some conclusions should be debated, such as, "This confirms the utility of FCM in enumerating CTCs and thus it can be used to monitor CH patients for early detection of HCC, being sensitive and easy, relatively less coasty, and more rapid compared to the currently used techniques such as PCR or Cell Search". From the results of this study, we cannot know CTCs is an early diagnosis or predict marker of HCC.

Authors Response: The conclusions have been corrected according to the final findings of the study with removal of the part concerning early detection.

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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