OPEN PEER REVIEW

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Peer Review Report for "Expression of CD28 in Hepatocellular Carcinoma and Its Prognostic Value"

Author(s): Xiaoke Ran, Chuanlei Zhang, Xinting Wang, Qing Zhao, Qiang Zhao, Changwei Yuan, Yuhui Kuang, Xiaoqi Chen, Xinju Chen

Review Timeline:

Submit Date: 14 Aug 2021
Revised Date: 19 Jan 2022
Accepted Date: 19 Jan 2022

Revision (0)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

Eisa Tahmasbpour: Reviewer | Revision (0) 26 Sep 2021

Dear AE,

This study evaluated “the expression of CD28 in hepatocellular carcinoma and its clinical significance”; however, the following queries should be clarified before a decision:

1. There are some grammatical errors throughout the manuscript that need to be revised.
2. In the abstract, you didn’t mention clinical outcomes, as well as the relationship between CD28 and clinical outcomes. If you considered only mortality or survival rate, the manuscript title should be limited to survival rate but not clinical outcome!
3. In the abstract you could bring a sentence to highlight CD28 as the study background.
4. It would be well if you could write some of main findings (e.g. fold-change ratio of CD28 expression in patients vs. controls) as quantitative or mean ±SD within the abstract/result.
5. What is the novelty of your work? You could mention it within the introduction.
6. The materials & methods section is relatively immature. You could expand it a bit more clearly for readers. For example, write about the sample size. How did you calculate sample

size for this survey? Furthermore, write about all applied exclusion and inclusion criteria a bit more clearly by which you selected patients for this survey.
7. It would be if you could summarize basic demographic and clinical information of all patients (e.g. age, height, weight, BMI, tumor size, underlying diseases, type of drug therapies, disease duration, history of chemotherapy, Systolic and Diastolic Pressures, history of serious diseases and their durations, and etc.) in a Table.
8. Write the name of all applied apparatuses, chemicals, kits, solutions you applied for biochemical and the other examinations. You should also mention the name of all companies you purchased materials from them.
9. Show abnormalities in microscopic images using some arrows.
10. Please show magnificent bar in microscopic figures.
11. Error bars in bar charts should be explained whether they are standard error bars (SE) or standard deviation (SD)?
12. A figure showing the flow-cytometry analysis of CD4+ and CD8+ cells is mandatory.
13. Why didn't you measure the relative expression or fold change ratio, as well? It would strengthen your data if you could determine the amount of fold-change ratio of CD28 expression.
14. Why didn't you collect blood samples from healthy individuals to perform a comparison between patients and controls?
15. Discussion section is still immature. You should expand it a bit more. You could increase the number of more recently published studies in the reference section. You should have comprehensive and reliable comparisons between your findings with the other previous studies. Furthermore, write about the limitations of your survey. Are there any limitations for this study? If yes, please mention all limitations of current study within the discussion section, too. Also, don't repeat the result section within the discussion section.

Regards

REFEREE: Reviewer | **Revision** (0) 6 Oct 2021

Dear editor,

The work is a preliminary study in discovering the relationship between CD28 and the CD4+ and CD8+ immune cells in HCC and the influence of these factors and their relationship on the survival of patients, but the Authors could not illustrate this hypothesis well and complete

1. The fact "CD28 plays 50 a regulatory role in tumor immunity by interacting with CD4 and CD8" which discussed in introduction, was not clarified and more related information should be added in introduction and more discussion regarding the results of this study.

2. Figures are not numbered and presented completely.

3. Results of FCM analysis on CD4 and CD8 cells were not presented.

4. The figure that showed semi-quantitative analysis of CD28 expression needs revision.

5. Define what IOD and area presented.

6. As the negative group actually had zero value and can not be displayed in chart, what would be the importance of its presentation?

7. The importance of AFP (alpha fetoprotein) and the related methods and results (which presented in table 1) were not discussed in text

Thanks

Mohammad Heiat: Reviewer | **Revision** (0) 6 Oct 2021

Dear E.I.C

I finalized the reviewing process right now.
Following points are notable as a major revision.
Abstract:
1. The data would be better to present quantitatively
2. A minimal reference should be made to the relationship between CD28 and CD4+ and CD8+ cell markers in the abstract.
Key word:
1. Arrange alphabetically with capital first letter.
Introduction:
1. More details about the molecular mechanism of cancer cells and T-cell in strongly recommended.
Methods:
1. What guarantee is there that the dysregulation of CD28 is related to HCC not to HBV infection? (As the patient with the history of hepatitis B have been included).
2. “The Barcelona Clinical Liver 63 Cancer (Barcelona Clinical Liver Cancer, BCLC) staging system” . This statement needs a reference.
3. “streptavidin-perosidase”??? OR “streptavidin-peroxidase”??
4. “FACS lysing solution” and its perfect protocol need a reference.
5. “Based on the median of CD28 expression level, tumor 96 samples were divided into high and low expression group for survival curve analysis.” What was the cut off (fold change) for dividing samples into two groups?
6. How the AFP (alpha fetoprotein) is measured is not mentioned.
7. The data gathering (Or excremental design) for survival analysis has not been mentioned. It should be totally explained.
Result:
1. The order of figures should be revised (Fig 1 is in the second place).
2. Fig 2. The zero point of the graph should be started from a higher position on Y axis that the bar of the negative expression be seen.
3. What is the Y axis unit in Fig 2. And by which means it is measured.
4. How the CD28 expression was significantly correlated with the degree of tumor differentiation but not with TNM stage. These two variables are strongly correlated.

5. That the Wilcoxon signed rank test has been used for correlation is not discussed
6. The cohort time for survival analysis is missed; this analysis is associated with some vague issues. E.g., all patient have been enrolled in the same time?, all patient followed similarly, maybe time-person method should be observed in this regard.
7. Tables are not well organized.
8. The figure legends as well as tables' are not perfect.
Discussion:
The author should discuss about their unilateral vision on cancer as the most concerning point.

Regards

Mohammad Heiat: Associate Editor | **Revision** (0) 6 Oct 2021

Dear EIC,

This manuscript needs Major Revision.

Thanks

Seyed Moayed Alavian: EIC | **Revision** (0) 6 Oct 2021

Dear Authors,

Thank you for your submission. Please follow the peer-review comments and submit a revision.

Thanks
EIC

OPEN PEER REVIEW

Revision (1)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear editor,

Thanks for your kind letter about our manuscript. The manuscript has been further polished according to the reviewers' comments and suggestion. We would like to resubmit the revised manuscript and hope it is acceptable now. Thanks for editor and reviewers' careful review of our manuscript. The revised text has been highlighted in the manuscript. The point-to-point response to comments are listed below. Don't hesitate to tell us, if there are additional questions.

Sincerely yours,

Xinju Chen

Reviewers' Comments:

Reviewer 1:

The work is a preliminary study in discovering the relationship between CD28 and the CD4+ and CD8+ immune cells in HCC and the influence of these factors and their relationship on the survival of patients, but the Authors could not illustrate this hypothesis well and complete.

Comment 1 The fact "CD28 plays a regulatory role in tumor immunity by interacting with CD4 and CD8" which discussed in introduction, was not clarified and more related information should be added in introduction and more discussion regarding the results of this study.

Response: According to your kind suggestion, we have added more information about the interaction between CD28 and CD4 and CD8 in the introduction section.

Figures are not numbered and presented completely.

Response: Thanks for your kind suggestion. Based on the results of our study, we have presented the figures that meet the requirements.

Results of FCM analysis on CD4 and CD8 cells were not presented

Response: Thanks for your constructive suggestion. We have added the results of FCM analysis in the results section, such as "The number of CD4+ and CD8+ cells in each blood sample of HCC patients were analyzed by flow cytometry. Results showed

that the count of CD4+ cells was 526.13 ± 258.17 cells/μl and the CD8+ cell count was 383.93 ± 223.39 cells/μl." Moreover, we have provided the corresponding figures.

The figure that showed semi-quantitative analysis of CD28 expression needs revision. Define what IOD and area presented. As the negative group actually had zero value and can not be displayed in chart, what would be the importance of its presentation?

Response: Thanks for your constructive suggestion. We have revised the figure of semi-quantitative analysis of CD28 expression. The IOD indicates integrated optical density and area means the CD28 positive staining percentage. We have defined IOD and area in the text, when first mentioned. Our study was aimed to evaluate the CD28 expression and its correlation with prognosis of HCC patients. The expression of CD28 in HCC patients were varied. Based on IOD value, sections were divided into negative group (IOD<1000), low expression group (IOD range: 10000-250000), and high expression group (IOD range: 250000-450000). Thus, IOD or area value in negative group is not zero and can be displayed in chart.

The importance of AFP (alpha fetoprotein) and the related methods and results (which presented in table 1) were not discussed in text.

Response: Thanks for your constructive suggestion. We have added the AFP related methods and results. The importance of AFP was discussed in the text.

Reviewer 2:

This study evaluated "the expression of CD28 in hepatocellular carcinoma and its clinical significance"; however, the following queries should be clarified before a decision:

1.There are some grammatical errors throughout the manuscript that need to be revised.

Response: According to your kind suggestion, we have carefully checked the whole text to avoid grammatical errors.

2. In the abstract, you didn't mention clinical outcomes, as well as the relationship between CD28 and clinical outcomes. If you considered only mortality or survival rate, the manuscript title should be limited to survival rate but not clinical outcome.

Response: Thanks for your constructive suggestion. We have revised the title related with the prognosis of HCC, such as "**The expression of CD28 in hepatocellular carcinoma and its prognostic value".**

3. In the abstract you could bring a sentence to highlight CD28 as the study background.

Response: According to your kind suggestion, we have added the study background about the role of CD28 in cancer, such as "CD28 expression is correlated with malignancies development in long-term survivors after liver transplantation."

4.It would be well if you could write some of main findings (e.g. fold-change ratio of CD28 expression in patients vs. controls) as quantitative or mean ±SD within the abstract/result.

Response: Thanks for your kind suggestion. We are sorry for failing to enroll controls in our study. But we have added detailed values about CD28 expression and the number of CD4+/CD8+ cells in the abstract and results.

5.What is the novelty of your work? You could mention it within the introduction.

Response: Thanks for your kind suggestion. CD28 expression has been reported to be the predictive marker for malignancies in survivors of liver transplantation. Besides, the activation of cytotoxic T-lymphoma (CTL) was mediated by the interactions between CD28 and T cell receptors (including CD4 and CD8) [9]. However, the relationship between CD28 and CD4+ and CD8+ immune cells in HCC and the prognostic value of CD28 expression have not been clarified. Thus, we conducted this study to explore it. We have widely revised the introduction section and highlighted the novelty of our work.

6.The materials & methods section is relatively immature. You could expand it a bit more clearly for readers. For example, write about the sample size. How did you calculate sample size for this survey? Furthermore, write about all applied exclusion and inclusion criteria a bit more clearly by which you selected patients for this survey.

Response: Thanks for your kind suggestion. For the data of CD28 expression and CD4 +/CD8+ cell count were rare, we did not find the reference value to perform

power analysis for calculating sample size. As shown in the previous study [9], the sample size was 20 for HCC patients and 15 for controls, which was comparable to the sample size of our study. Between January 2014 and July 2019, all the HCC patients met the inclusion and exclusion criterion were included in our study. The inclusion criteria included that HCC determined based on pathological diagnosis; patients with the history of hepatitis B; patients had not received any treatment before this study. The exclusion criterion was that patients with the history of HBV infection. The detailed exclusion and inclusion criteria have been added in the manuscript.

7. It would be if you could summarize basic demographic and clinical information of all patients (e.g. age, height, weight, BMI, tumor size, underlying diseases, type of drug therapies, disease duration, history of chemotherapy, Systolic and Diastolic Pressures, history of serious diseases and their durations, and etc.) in a Table.

Response: According to your kind suggestion, we have summarized the basic demographic and clinical information of patients with regard to age, tumor size, number of tumors, TNM stage, differentiation, metastasis and symptoms in a Table defined as Table 1.

8. Write the name of all applied apparatuses, chemicals, kits, solutions you applied for biochemical and the other examinations. You should also mention the name of all companies you purchased materials from them.

Response: According to your kind suggestion, we have added the manufacturer information of the applied apparatuses, chemicals, kits as much as possible.

9.Show abnormalities in microscopic images using some arrows.

Response: Thanks for your kind suggestion. We have added the arrows to indicate CD28 expression in Figure 1.

10. Please show magnificent bar in microscopic figures.

Response: Thanks for your kind suggestion. We have added the scale bar in figure 1.

11.Error bars in bar charts should be explained whether they are standard error bars (SE) or standard deviation (SD)?

Response: Thanks for your constructive suggestion. The quantitative data were expressed as mean ±standard deviation (SD) and we have added this point in method section. Thus, the error bars in figures are standard deviation (SD).

12.A figure showing the flow-cytometry analysis of CD4+ and CD8+ cells is mandatory.

Response: Thanks for your kind suggestion. We have provided the images of flow-cytometry analysis in the results section. Besides, we have added the quantitative data of flow-cytometry analysis in the result section, such as “The number of CD4+ and CD8+ cells in each blood sample of HCC patients were analyzed by flow cytometry. Results showed that the count of CD4+ cells was 526.13 ± 258.17 cells/μl and the CD8+ cell count was 383.93 ± 223.38 cells/μl.”

13.Why didn’t you measure the relative expression or fold change ratio, as well? It would strengthen your data if you could determine the amount of fold-change ratio of CD28 expression.

Response: Thanks for your constructive suggestion. In this study, we did not collect the healthy subjects for controls, but the patients with negative expression of CD28 was set as the controls. For healthy controls, there were no abnormal results for pathological test, and serum test. Maybe, we could just provide the pathological images for adjacent normal tissues. Our study was aimed to evaluate the varied expression of CD28 in HCC patients. Thus, we could not determine the fold change of CD28 expression. It may be a limitation in our study, we have added in the manuscript.

14.Why didn’t you collect blood samples from healthy individuals to perform a comparison between patients and controls?

Response: Thanks for your constructive suggestion. We are sorry for the limitation of not including the healthy controls in our study. It is a limitation in our study and we have added in discussion section.

15. Discussion section is still immature. You should expand it a bit more. You could increase the number of more recently published studies in the reference section. You should have comprehensive and reliable comparisons between your findings with the

other previous studies. Furthermore, write about the limitations of your survey. Are there any limitations for this study? If yes, please mention all limitations of current study within the discussion section, too. Also, don't repeat the result section within the discussion section.

Response: According to your kind suggestion, we have widely revised the discussion section and added other previous studies. The limitations have been written in end of discussion section.

Reviewer 3:

Dear E.I.C

I finalized the reviewing process right now.

Following points are notable as a major revision.

Abstract:

1.The data would be better to present quantitatively

Response: Thanks for your kind suggestion. We have added the quantitative data in the abstract.

2.A minimal reference should be made to the relationship between CD28 and CD4+ and CD8+ cell markers in the abstract.

Response: According to your kind suggestion, we have added the background about the relationship between CD28 and CD4 and CD8, such as "CD28 expression is correlated with malignancies development in long-term survivors after liver transplantation. The immune cell activation is mediated by the interaction between CD28 with CD4 and CD8."

Key word: Arrange alphabetically with capital first letter.

Response: According to your kind suggestion, we have re-arranged the keywords alphabetically with capital first letter.

Introduction:

1.More details about the molecular mechanism of cancer cells and T-cell in strongly recommended.

Response: Thanks for your kind suggestion. We have carefully revised the introduction section and added the information of the interaction between CD28 with CD4 and CD8 in the introduction section.

Methods:

1.What guarantee is there that the dysregulation of CD28 is related to HCC not to HBV infection? (As the patient with the history of hepatitis B have been included).

Response: Thanks for your kind suggestion. Patients with the history of hepatitis B was one of the inclusion criterion in our study and we have added this information in the method section.

2.“The Barcelona Clinical Liver 63 Cancer (Barcelona Clinical Liver Cancer, BCLC) staging system” . This statement needs a reference.

Response: According to your kind suggestion, we have added a reference for BCLC staging system in the manuscript.

3.“streptavidin-perosidase”??? OR “streptavidin-peroxidase”??

Response: Thanks for your kind suggestion. We have revised “streptavidin-perosidase” to “streptavidin-peroxidase”.

4.“FACS lysing solution” and its perfect protocol need a reference.

Response: According to your kind suggestion, we have added the reference for the protocol of “FACS lysing solution”.

5.“Based on the median of CD28 expression level, tumor 96 samples were divided into high and low expression group for survival curve analysis.” What was the cut off (fold change) for dividing samples into two groups?

Response: The samples were divided into high and low CD28 expression group based on the median expression value of 68.95, which had been mentioned in the method section.

6.How the AFP (alpha fetoprotein) is measured is not mentioned.

Response: Thanks for your kind suggestion. We have added related information in the method section, such as “The blood samples of included patients were collected at

diagnosis and the serum AFP level was detected by chemiluminescence immunoassay kit (Mlbio, Shanghai, China) and detected by automatic chemiluminescence analyzer (DX1800, Beckman Coulter, Inc., Fullerton, CA).”

7.The data gathering (Or excremental design) for survival analysis has not been mentioned. It should be totally explained.

Response: Thanks for your kind suggestion. We have added the information of data gathering for survival analysis in the method section, such as “All the included patients were followed up for the following 5 years. The survival data were collected during hospitalized period or by telephone contact post-discharge.”

Result:

1.The order of figures should be revised (Fig 1 is in the second place).

Response: Thanks for your kind suggestion. We have carefully checked the order of figures and confirmed that it is right.

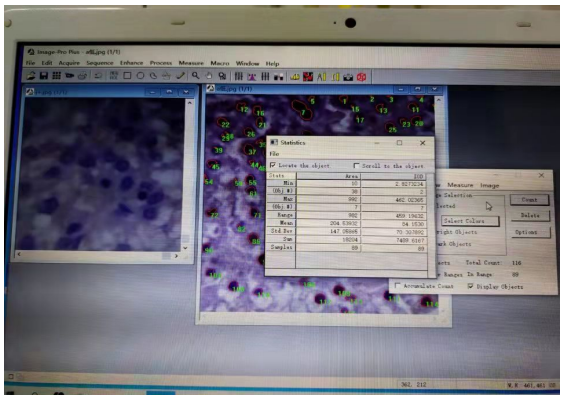
2.Fig 2. The zero point of the graph should be started from a higher position on Y axis that the bar of the negative expression be seen.

Response: Thanks for your kind suggestion. We have revised figure 2 according to your kind suggestion.

3.What is the Y axis unit in Fig 2. And by which means it is measured.

Response: Thanks for your kind suggestion. The area and IOD value were measured by using Image-Pro Plus software and there were no unit for area and IOD value.

The output page is listed below.



4.How the CD28 expression was significantly correlated with the degree of tumor differentiation but not with TNM stage. These two variables are strongly correlated.

Response: Patients with low differentiation may have poor prognosis, while those with high differentiation may have good prognosis. Thus, there was no direct correlation between degree of tumor differentiation and TNM stage.

5.That the Wilcoxon signed rank test has been used for correlation is not discussed.

Response: Thanks for your kind suggestion. We have widely revised the discussion section and added the discussion about the results of correlation analysis.

6.The cohort time for survival analysis is missed; this analysis is associated with some vague issues. E.g., all patient have been enrolled in the same time?, all patient followed similarly, maybe time-person method should be observed in this regard.

Response: Thanks for your kind suggestion. From January 2014 to July 2019, patients with HCC were recruited from our hospital. Patients meeting the inclusion and exclusion criterion were included. Then, the eligible patients were followed up for 5 years by telephone contact. We have added this information in the method section.

7.Tables are not well organized.

Response: Thanks for your kind suggestion. We have added the table for the baseline characteristics of included patients and revised the tables.

8.The figure legends as well as tables' are not perfect.

Response: Thanks for your kind suggestion. We have added a table for the basic information of included patients and the figure legends are carefully revised.

Discussion:

The author should discuss about their unilateral vision on cancer as the most concerning point.

Response: According to your kind suggestion, we have widely revised the discussion section.

OPEN PEER REVIEW

Revision (1)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

Eisa Tahmasbpour: Reviewer | **Revision** (1) 16 Dec 2021

Dear editor,

The authors have revised the manuscript accordingly; however, lack of control group is a major concern for this study. They might mention this limitation in the conclusion or discussion section.

Thanks

Mohammad Heiat: Reviewer | **Revision** (1) 27 Dec 2021

Dear EIC
Authors have addressed all comment in a proper way. The manuscript now is ready for future proceeding.
Thanks
Dr. M.Heiat

Mohammad Heiat: Associate Editor | **Revision** (1) 27 Dec 2021

Dear EIC,

The manuscript needs Minor Revision.

Regards

Seyed Moayed Alavian: EIC | **Revision** (1) 27 Dec 2021

Dear Authors,

Thank you for submitting the revised manuscript. The peer-review team has asked for additional corrections before accepting your work.

Regards,
EIC

OPEN PEER REVIEW

Revision (2)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear Editor

We hope that the manuscript NO. 118605 entitled "Expression of CD28 in Hepatocellular Carcinoma and Its Clinical Significance" will be considered for publication in your journal. There is no conflict of interest in the submission of this manuscript, and the manuscript is published with the consent of all authors.

Thanks to the reviewers for their comments, the revised part has been revised in the article and highlighted in blue. If you have any questions, please don't hesitate to contact me at the address below.

Thank you and best wishes.

Yours sincerely

OPEN PEER REVIEW

Revision (2)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

Seyed Moayed Alavian: EIC | **Revision** (2) 19 Jan 2022

Dear Authors,

I am delighted to inform that your manuscript is accepted for publication in Hepatitis Monthly journal.

Regards,
EIC