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PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology

Manuscript NO: 48265

Title: Abnormal CD44 Activation of Hepatocytes with Nonalcoholic Fatty Accumulation in Rat Hepatocarcinogenesis

Reviewer's code: 00073640

Reviewer's country: Slovenia

Science editor: Jia-Ping Yan

Reviewer accepted review: 2019-04-15 07:02

Reviewer performed review: 2019-04-16 06:33

Review time: 23 Hours

SCIENTIFIC QUALITY	LANGUAGE QUALITY	CONCLUSION	PEER-REVIEWER STATEMENTS
[ ] Grade A: Excellent	[ ] Grade A: Priority publishing	[ ] Accept (High priority)	Peer-Review:
[ ] Grade B: Very good	[ ] Grade B: Minor language polishing	[ ] Accept (General priority)	[Y] Anonymous
[ ] Grade C: Good	[Y] Grade C: A great deal of language polishing	[ ] Minor revision	[ ] Onymous
[Y] Grade D: Fair	[ ] Grade D: Rejection	[Y] Major revision	Peer-reviewer's expertise on the topic of the manuscript:
[ ] Grade E: Do not publish		[ ] Rejection	[Y] Advanced
			[ ] General
			[ ] No expertise
			Conflicts-of-Interest:
			[ ] Yes
			[Y] No

SPECIFIC COMMENTS TO AUTHORS

The title/subject is very interesting and topical, the manuscript is well structured. However, it is very difficult to read the manuscript because of English, which needs significant corrections. In addition, there are important issues which should be

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addressed in the manuscript very clearly. Authors conducted experiment on animals, thus they should include the following information. Material and method section: - Information about the approval of the animal experiment and licence number - Information about the origin of the rats - Explanation, why the number of the rats in 5 groups were different (in the results section there are data: control group n= 12, NAFLD group n=24, hepatocyte damage n=17, precancerosis n=15, HCC formation n=10) - Information, who made the diets (NAFLD and 2-FAA) and in what form the diet was given to rats (manufacturer, code of the diet, pellets etc). What is 75% common diet - authors should state manufacturer, code of the diet. Since diet is in this experiment very important data they should state all the information about the preparation of the diet- who mixed the diets and prepared both diets, consumption of the diet intake during experiment etc. - The design of the experiment is not clear. Authors wrote that animals received high fat diet for 2 weeks and then high fat diet plus 2-FAA (for inducing HCC formation) and then rats were sacrificed every 2 weeks. I strongly suggest explaining the design of the experiment and include schematic presentation of the design. - Authors should also state the microbiological state of the animals (health monitoring report), because there are a number microorganisms in rodents that do not cause clinical disease but can significantly affect the liver pathology and consequently affect the reproducibility and validity of the study - What were the housing conditions – how were animals housed (in groups, singly), bedding material, space, enrichment? All these information can affect the animal health and consequently the results and affect reproducibility and validity of the study - Liver tissues and Serum samples sections should follow the fatty accumulated HCC model section and should state, what they measured in liver and serum – then should follow histological and biochemical analyses. It is strange that authors used more space for the explanation of the simple standard methods of histology than explanation of the animal experiment, which is much more

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complicated and had numerous factors that can affect results and thus should be described. - Since authors measured the lipid profile in the blood, they should also state the time of the killing of animals for all groups as well as information about the potential fasting of animals (circadian rythms of cholesterol levels may affect the results significantly). - Authors wrote that they investigated different stages of HCC development – i.e. "hepatocytes damage (denaturation), the precancerosis and HCC formation". Authors explained that the liver were assessed histologically (HE staining) but did not explain the criteria for all the stages (control, NAFLD, denaturation, precancerousis, HCC) and how they evaluate these stages. I am a pathologist and I am very curious how the authors assessed hepatocyte damage, precancerosis and HCC formation stages? The term denaturation is not correct!!??? - Authors used also OilRed staining to evaluate the lipid droplets (macro or micovesicular statosis). Thus, if there is lipid accumulation in the liver tissue big red droplets should be seen (i.e. macrovesicular steatosis) or numerous small red droplets should be seen in the hepatocytes (i.e. microvesicular steatosis). Instead, in the figure 1b,c,d I can see only red color without any shape, suggesting that the staining with OirRed was not performed correctly. Figure 1e shows microvescular statosis. - Likewise, the figures of the immunohistological staining (Figure 2) show, that the staining was correctly performed - it is non- specific, there are background and artefacts. - The use of IOD values in such cases is usesless. I strongly suggest including the experienced pathologist in the study to evaluate the stages of hepatocarcinogenesis, the statosis (micro or macrovesicular) and the immunostaning. - In the results section you should include the data about the health of the rats, their body weight during experiment, the weight of the organs (liver, spleen, kidney) and the weight of the visceral fat of rats at the authopsy – these data are necessary in animal experiments

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INITIAL REVIEW OF THE MANUSCRIPT

Google Search:

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Thanks to Reviewer 1 for your very kindly comments.

The title/subject is very interesting and topical, the manuscript is well structured. However, it is very difficult to read the manuscript because of English, which needs significant corrections. In addition, there are important issues which should be addressed in the manuscript very clearly. Authors conducted experiment on animals, thus they should include the following information. Material and method section: - Information about the approval of the animal experiment and licence number - Information about the origin of the rats –

1. Explanation, why the number of the rats in 5 groups were different (in the results section there are data: control group n= 12, NAFLD group n=24, hepatocyte damage n=17, precancerosis n=15, HCC formation n=10)
   They were divided into groups only dependence on pathological examnation.

2. Information, who made the diets (NAFLD and 2-FAA) and in what form the diet was given to rats (manufacturer, code of the diet, pellets etc).
   Based on reported of previously articles.

3. What is 75% common diet - authors should state manufacturer, code of the diet. Since diet is in this experiment very important data they should state all the information about the preparation of the diet- who mixed the diets and prepared both diets, consumption of the diet

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intake during experiment etc.
Based on reported of previously articles.

4. The design of the experiment is not clear. Authors wrote that animals received high fat diet for 2 weeks and then high fat diet plus 2-FAA (for inducing HCC formation) and then rats were sacrificed every 2 weeks. I strongly suggest explaining the design of the experiment and include schematic presentation of the design.
   Based on reported of previously articles.

5. Authors should also state the microbiological state of the animals (health monitoring report), because there are a number microorganisms in rodents that do not cause clinical disease but can significantly affect the liver pathology and consequently affect the reproducibility and validity of the study.
   Thanks to you, next time, we should examine the microbiological state of the animals (health monitoring report).

6. What were the housing conditions – how were animals housed (in groups, singly), bedding material, space, enrichment? All these information can affect the animal health and consequently the results and affect reproducibility and validity of the study.
   OK, no problem.

7. Liver tissues and Serum samples sections should follow the fatty accumulated HCC model section and should state, what they measured in liver and serum – then should follow histological and biochemical analyses. It is strange that authors used more space for the explanation of the simple standard methods of histology than explanation of the animal experiment, which is much more complicated and had numerous factors that can affect results and thus should be described.

OK, no problem.

8. Since authors measured the lipid profile in the blood, they should also state the time of the killing of animals for all groups as well as information about the potential fasting of animals (circadian rythms of cholesterol levels may affect the results significantly).
   Thanks to you, next time, we should pay attendation on it.

9. Authors wrote that they investigated different stages of HCC development – i.e. “hepatocytes damage (denaturation), the precancerosis and HCC formation”. Authors explained that the liver were assessed histologically (HE staining) but did not explain the criteria for all the stages (control, NAFLD, denaturation, precancerousis, HCC) and how they evaluate these

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stages. I am a pathologist and I am very curious how the authors assessed hepatocyte damage, precancerosis and HCC formation stages? The term denaturation is not correct!!???

According to the previously reported articles, we divided the rats into different stages.

10. Authors used also Oil Red staining to evaluate the lipid droplets (macro or micovesicular statosis). Thus, if there is lipid accumulation in the liver tissue big red droplets should be seen (i.e. macrovesicular steatosis) or numerous small red droplets should be seen in the hepatocytes (i.e. microvesicular steatosis). Instead, in the figure 1b,c,d I can see only red color without any shape, suggesting that the staining with Oir Red was not performed correctly. Figure 1e shows microvescular statosis.

    According to the previously reported articles.

11. Likewise, the figures of the immunohistological staining (Figure 2) show, that the staining was correctly performed - it is non-specific, there are background and artefacts.

    Those showed the results of IHC staining.

12. The use of IOD values in such cases is usesless. I strongly suggest including the experienced pathologist in the study to evaluate the stages of hepatocarcinogenesis, the statosis (micro or macrovesicular) and the immunostaning.

    Thanks to you, we should examine the IOD values for comparative analysis.

13. In the results section you should include the data about the health of the rats, their body weight during experiment, the weight of the organs (liver, spleen, kidney) and the weight of the visceral fat of rats at the authopsy – these data are necessary in animal experiments。

Thanks to you, next paper, we should report more in dital detection about the weight of all rats' organs.

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PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology

Manuscript NO: 48265

Title: Abnormal CD44 Activation of Hepatocytes with Nonalcoholic Fatty Accumulation in Rat Hepatocarcinogenesis

Reviewer's code: 02440884

Reviewer's country: Germany

Science editor: Jia-Ping Yan

Reviewer accepted review: 2019-04-15 20:50

Reviewer performed review: 2019-04-16 20:40

Review time: 23 Hours

SCIENTIFIC QUALITY	LANGUAGE QUALITY	CONCLUSION	PEER-REVIEWER STATEMENTS
[ ] Grade A: Excellent [ ] Grade B: Very good [Y] Grade C: Good [ ] Grade D: Fair [ ] Grade E: Do not publish	[ ] Grade A: Priority publishing [ ] Grade B: Minor language polishing [Y] Grade C: A great deal of language polishing [ ] Grade D: Rejection	[ ] Accept (High priority) [ ] Accept (General priority) [ ] Minor revision [Y] Major revision [ ] Rejection	Peer-Review: [Y] Anonymous [ ] Onymous Peer-reviewer's expertise on the topic of the manuscript: [Y] Advanced [ ] General [ ] No expertise Conflicts-of-Interest: [ ] Yes [Y] No

SPECIFIC COMMENTS TO AUTHORS

In the experimental study the role of CD 44 in carcinogenesis of hepatocellular carcinoma was addressed. A rat model of NAFLD (high fat diet) and HCC induction with 2-fluorenylacetamide was used. The authors demonstrate data indicating that an

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increasing CD44 expression could be associated with the malignant transformation of hepatocytes in NAFLD. Comments 1. Histological evidence for severe NAFLD is given. The degree of inflammation should be addressed. 2. The authors claim that CD44 is involved with carcinogenesis. To further substantiate the data a more in-detail analysis of liver tissues is necessary. Is there any evidence for a low-/ or high-grade dysplastic nodule with accumulation of CD44? 3. The cell types expressing CD44 should be identified. Is there any evidence for CD44 synthesis by stellate cells?

INITIAL REVIEW OF THE MANUSCRIPT

Google Search:

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[ ] Duplicate publication

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[ ] No

BPG Search:

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[ ] Duplicate publication

[ ] Plagiarism

[ ] No

Thanks to you for your very kindly comments.

In the experimental study the role of CD44 in carcinogenesis of hepatocellular carcinoma was addressed. A rat model of NAFLD (high fat diet) and HCC induction with 2-fluorenylacetamide was used. The authors demonstrate data indicating that an increasing CD44 expression could be associated with the malignant transformation of hepatocytes in NAFLD. Comments

1. Histological evidence for severe NAFLD is given. The degree of inflammation should be addressed.

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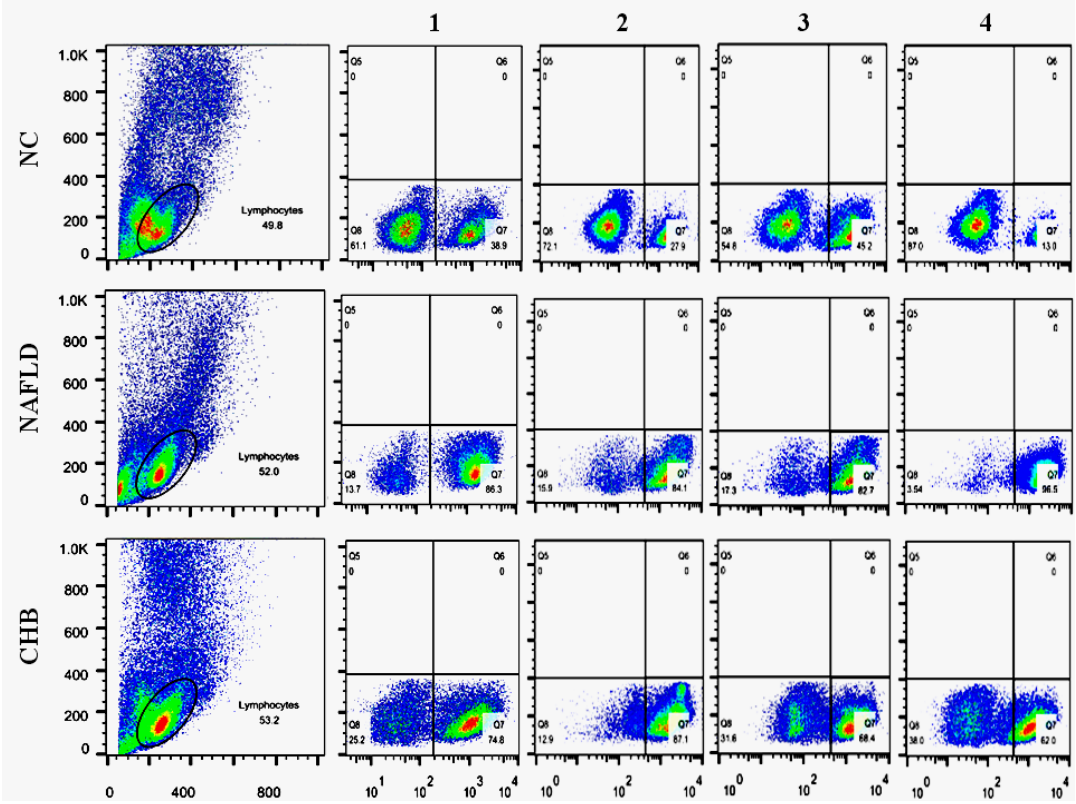
Some biomarkers for hepatocytes damages have been detected such as ALT and AST activities with significantly increasing during rat models for HCC inducing.

2. The authors claim that CD44 is involved with carcinogenesis. To further substantiate the data a more in-detail analysis of liver tissues is necessary. Is there any evidence for a low-/ or high-grade dysplastic nodule with accumulation of CD44?

Thanks to you for your suggestions. In this MS, there isn't any evidence for a low-/ or high-grade dysplastic nodule with accumulation of CD44.

3. The cell types expressing CD44 should be identified. Is there any evidence for CD44 synthesis by stellate cells?

Now, we haven't any evidence for CD44 synthesis by stellate cells. However, about CD44 investigation in patients with NAFLD showed the significantly higher jast as followed Figure (not published data) and the percentage of CD44 positive lymphocytes in peripheral blood of patients with NAFLD (t= 7.691, P<0.05) and cases with chronic hepatitis B (t=3.852, P<0.05) was higher than that in health control group. And it was even higher in NAFLD comparing with CHB (t=2.045, P=0.045).



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PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology

Manuscript NO: 48265

Title: Abnormal CD44 Activation of Hepatocytes with Nonalcoholic Fatty Accumulation in Rat Hepatocarcinogenesis

Reviewer's code: 02451459

Reviewer's country: Reviewer_Country

Science editor: Jia-Ping Yan

Reviewer accepted review: 2019-04-16 23:26

Reviewer performed review: 2019-04-19 07:49

Review time: 2 Days and 8 Hours

SCIENTIFIC QUALITY	LANGUAGE QUALITY	CONCLUSION	PEER-REVIEWER STATEMENTS
[ ] Grade A: Excellent [Y] Grade B: Very good [ ] Grade C: Good [ ] Grade D: Fair [ ] Grade E: Do not publish	[ ] Grade A: Priority publishing [Y] Grade B: Minor language polishing [ ] Grade C: A great deal of language polishing [ ] Grade D: Rejection	[ ] Accept (High priority) [ ] Accept (General priority) [Y] Minor revision [ ] Major revision [ ] Rejection	Peer-Review: [Y] Anonymous [ ] Onymous Peer-reviewer's expertise on the topic of the manuscript: [ ] Advanced [Y] General [ ] No expertise Conflicts-of-Interest: [ ] Yes [Y] No

SPECIFIC COMMENTS TO AUTHORS

General comments: This is a longitudinal study to establish the correlation between CD44 and the progression of NAFLD towards HCC. By using SD rats fed with high fat diet, followed by administration of 2-fluorenyl-acetamide to induce malignant

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transformation, the expression of CD44 in the liver and serum were tracked and compared against liver injury markers and AFP. While there is clear association between CD44 and disease progression, this study did not allude to a cause and effect relationship. The potential of CD44 as a therapeutic target to arrest disease progression from NAFLD would require much more investigation. 1. It is known that not all NAFLD will progress into HCC. Therefore, if a high CD44 is seen at the early stage of NAFLD, it may not present prognostic value. Further study to indicate a cause and effect relationship would be important. I.e., does the silencing of CD44 blocks disease progression? 2. The description of the hepatocyte lipid content in the different treatments, do not correlate with the profile shown in Figure 1F. Figure 1F shows that NAFLD (b) and HCC (e) have the highest lipid content but the description in the maintext said that the precancerosis has the highest instead. 3. Please explain why different sample size was applied across the different arms of the experiment, ranging from n = 10 (for HCC arm) to n =24 (for NAFLD).

INITIAL REVIEW OF THE MANUSCRIPT

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[ ] No

Thanks to Reviewer 3 for your very kindly comments and the mistake has been corrected.

This is a longitudinal study to establish the correlation between CD44 and the progression of NAFLD towards HCC. By using SD rats fed with high fat diet, followed by administration of 2-fluorenyl-acetamide to induce malignant transformation, the expression of CD44 in the liver and serum were tracked and compared against liver injury markers and AFP. While there is clear association between CD44 and disease progression, this study did not allude to a cause and effect relationship. The potential of CD44 as a therapeutic target to arrest disease progression from NAFLD would require much more investigation.

1. It is known that not all NAFLD will progress into HCC. Therefore, if a high CD44 is seen at the early stage of NAFLD, it may not present prognostic value. Further study to indicate a cause and effect relationship would be important. I.e., does the silencing of CD44 blocks disease progression?

   Yes, you are right and thanks to you. Further study, we should investigate the silencing of CD44 on effects of NAFLD to HCC.

2. The description of the hepatocyte lipid content in the different treatments, do not correlate with the profile shown in Figure 1F. Figure 1F shows that NAFLD (b) and HCC (e) have the highest lipid content but the description in the maintext said that the precancerosis has the highest instead.

   This is only relative quantiative CD44 values for comparative analysis among the different groups, Significant different by the IOD values of CD44 expression was found (Fig.2F) between the control group and the NAFLD (t = 25.433, P<0.001), or hepatocytes denaturation (t=48.822, P<0.001), pre- cancerosis (t =27.751, P<0.001), HCC (t=16.239, P<0.001) group, respectively.

3. Please explain why different sample size was applied across the different arms of the experiment, ranging from n = 10 (for HCC arm) to n =24 (for NAFLD).

   They were divided into groups only dependence on pathological examnation.

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PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology

Manuscript NO: 48265

Title: Abnormal CD44 Activation of Hepatocytes with Nonalcoholic Fatty Accumulation in Rat Hepatocarcinogenesis

Reviewer's code: 00697631

Reviewer's country: Reviewer_Country

Science editor: Jia-Ping Yan

Reviewer accepted review: 2019-04-16 11:03

Reviewer performed review: 2019-04-24 11:34

Review time: 8 Days

SCIENTIFIC QUALITY	LANGUAGE QUALITY	CONCLUSION	PEER-REVIEWER STATEMENTS
[ ] Grade A: Excellent [ ] Grade B: Very good [Y] Grade C: Good [ ] Grade D: Fair [ ] Grade E: Do not publish	[ ] Grade A: Priority publishing [Y] Grade B: Minor language polishing [ ] Grade C: A great deal of language polishing [ ] Grade D: Rejection	[ ] Accept (High priority) [ ] Accept (General priority) [ ] Minor revision [Y] Major revision [ ] Rejection	Peer-Review: [Y] Anonymous [ ] Onymous Peer-reviewer's expertise on the topic of the manuscript: [ ] Advanced [Y] General [ ] No expertise Conflicts-of-Interest: [ ] Yes [Y] No

SPECIFIC COMMENTS TO AUTHORS

RE: Manuscript NO: 48265 Fan M et al, Abnormal CD44 Activation of Hepatocytes with Nonalcoholic Fatty Accumulation in Rat Hepatocarcinogenesis Fan M et al examined the expression of CD44 that is regarded as a cancer stem cell (CSC) marker of HCC in

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NASH model over time. The authors described the changes of CD44 expression by measuring mRNA level, ELISA and immunohistochemical staining during the progression of NASH and hepatocarcinogenesis. The authors also showed the correlation between CD44 and AFP expression in the animal model. The data presented in the manuscript include interesting findings in the process of HCC development in the animal NASH model, however, there are serious concerns that should be addressed. 1. CD44 is a well established CSC marker of HCC, however recent studies reported that the expression of CD44 alone is not sufficient to account for all of the biological properties of CSCs (Salnikov AV et al, Cancer Lett 2009; 275:185). Why do the authors not examine the expression of another CSC marker of HCC, for example CD133 which is commonly used in combination with CD44? 2. The authors examined the expression AFP simultaneously and showed the positive correlation between CD44 and AFP expression. AFP is well known established marker of HCC, however, AFP is also produced by non-malignant liver progenitor cells. Therefore the authors should demonstrate the AFP-positive cells in the liver tissue and compare the distribution of CD44 and AFP to support the authors' conclusion. 3. The authors divided the liver disease stage like middle early stage and late stage. How long do the animals treated with high fat diet and/or 2-FAA? Specific period of treatment should be described. 4. There are typographical and grammatical errors throughout the manuscript. English should be checked by native speakers.

INITIAL REVIEW OF THE MANUSCRIPT

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Thanks to You for your very kindly comments.

RE: Manuscript NO: 48265 Fan M et al, Abnormal CD44 Activation of Hepatocytes with Nonalcoholic Fatty Accumulation in Rat Hepatocarcinogenesis Fan M et al examined the expression of CD44 that is regarded as a cancer stem cell (CSC) marker of HCC in NASH model over time. The authors described the changes of CD44 expression by measuring mRNA level, ELISA and immunohistochemical staining during the progression of NASH and hepatocarcinogenesis. The authors also showed the correlation between CD44 and AFP expression in the animal model. The data presented in the manuscript include interesting findings in the process of HCC development in the animal NASH model, however, there are serious concerns that should be addressed.

1. CD44 is a well established CSC marker of HCC, however recent studies reported that the expression of CD44 alone is not sufficient to account for all of the biological properties of CSCs (Salnikov AV et al, Cancer Lett 2009; 275:185). Why do the authors not examine the expression of another CSC marker of HCC, for example CD133 which is commonly used in combination with CD44?
   Thanks to you for your consideration. This paper was first time reported the CD44 alteration from NAFLD to HCC devoloping. Also, next works should continue to investigate the CD133 and other CSC markers.

2. The authors examined the expression AFP simultaneously and showed the positive correlation between CD44 and AFP expression. AFP is well known established marker of HCC, however, AFP is also produced by non-malignant liver progenitor cells. Therefore the authors should demonstrate the AFP-positive cells in the liver tissue and compare the distribution of CD44 and AFP to support the authors' conclusion.
   You are right; this is only for date analysis, and thanks to you.

3. The authors divided the liver disease stage like middle early stage and late stage. How long do the animals treated with high fat diet and/or 2-FAA? Specific period of treatment should be described.
   They have been described in parts of MATERIALS AND METHODS.

4. There are typographical and grammatical errors throughout the manuscript. English should be checked by native speakers.

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Thanks to you and the mistake has been corrected.

We also appreciate the reviewers' careful and thoughtful suggestions, since the comments are all valuable and helpful for improving our paper. We have studied comments and made modifications according to the reviewers' comments.

Thank you again for publishing our manuscript in the ***WJGO***.

Sincerely yours,

Deng-fu Yao, **MD, PhD, Professor**,

Research Center of Clinical Medicine,
Affiliated Hospital of Nantong University,
No. 20 West Temple Road,
Nantong 226001, Jiangsu Province,
China.
E-mail: yaodf@ahnmc.com
Telephone: +86-513-85052413
Fax: +86- 513-85052523

ANSWERING REVIEWERS

Sept 19, 2019

Dear Editor and Reviewer,

Please find enclosed the edited manuscript in Word format (**MS 48265R1**).

Title: **Abnormal CD44 activation of hepatocytes with nonalcoholic fatty accumulation in rat hepatocarcinogenesis**
Author: **Miao Fang, Min Yao, Jie Yang, Wen-Jie Zheng, Li Wang, Deng-Fu Yao**
Name of Journal: ***World Journal of Gastrointestinal Oncology***

We are truly grateful to your comments concerning our manuscript **MS. 48265R1**.

Reviewer 1 comments to the author
#Reviewer 1:

I have reviewed the responses from the authors. I am satisfied with the answers provided for my queries. Nothing further from me.

Thanks to Reviewer 1 for your very kindly comments.

#Reviewer 2 comments to the author

Thank you for the chance to re-evaluate the revised Version of the manuscript. There is some improvement, but the Investigation of liver histology does not satisfy (see Point 1. of the original Statement). The whole Statement to the R1 in the following: In the experimental study the role of CD 44 in carcinogenesis of hepatocellular carcinoma was addressed in a rat model of NAFLD (high fat diet) and HCC induction with 2-fluorenylacetamide.

In the revised version essential points are addressed. However, a scoring system to evaluate liver tissues concerning the degree of inflammation and fibrosis is not given. The surrogate markers of tissue damage, ALT and AST, are only given. To my opinion, theses markers are not sufficient to reflect NAFLD/ NASH.

Thanks to Reviewer 2' careful and thoughtful suggestions, since the comments are all valuable and helpful for improving our paper. We have studied comments and made modifications according to the reviewers' comments.

To the best of our knowledge, this is the first report to investigate the relationship between increasing CD44 activation and malignant transformation

of rat hepatocytes. The new findings are promising, and the initial evidence confirmed that hepatic CD44 is one of the early molecules from NAFLD to HCC progression. However, the investigation of liver histology did not analyze the relationship bewteen CD44 level and Liver fibrosis. Future studies should evaluate liver tissues concerning the degree of fibrosis and CD44 activation.

Sincerely yours,

Deng-fu Yao, **MD, PhD, Professor**,

Research Center of Clinical Medicine,
Affiliated Hospital of Nantong University,
No. 20 West Temple Road,
Nantong 226001, Jiangsu Province, China.

E-mail: yaodf@ahnmc.com
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