Dear Editor,

We are very pleased to learn from your letter about revision for our manuscript (Manuscript NO: 67616). Thank you very much for providing us the reviewers' suggestion and the opportunity for us to resubmit our revised manuscript.

We have revised the manuscript according to the comments from the reviewers and editor. Our responses to the reviewers' comments are described as follows, with revision tracks in the revised manuscript and supplementary material.

We hope that the revised manuscript could be considered acceptable for publication. We are looking forward to hearing from you.

Sincerely,

Thank you and all the best regards,

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Manuscript NO: 67616

Demonstration of a bi-specific T1 positive-contrast-enhanced magnetic resonance imaging molecular probe for hepatocellular carcinoma in an orthotopic mouse model

World Journal of Gastroenterology

The following peer-review report and comments from the Editorial Office (Science Editor, Editorial Office Director, and Company Editor-in-Chief) are provided for your reference.

1 Peer-review report

Reviewer #1: This is a well-written paper containing interesting results. For the benefit of readers, however, a number of points need clarifying and certain statements require further justification. These are given below.

1. How many samples were analyzed in this study? Error bars should be shown in Figure 2 (B, C, D) and Figure 5 (A, B)

Response:

We thank the reviewer for helping us strengthen the manuscript and we have analyzed the data again and added the possible error bars. The corresponding data analysis methods were also added in the "Materials and Methods" part.

Briefly, we have added error bars in Figure 2(B, D, E) and Figure 2(C) might not be suitable to show error bars. While, for Figure 5, as limited by the real situation of experiment, the tumor-to-background ratio of maximum two mice were analyzed, and we feel regret could not calculate the standard deviation. But according to the reviewer's kind suggestion, we tried to use multiple measurement during MRI intensity analysis to guarantee the reliability of the data results. The detailed methods and results for revising the Figure 2 and Figure 5 were described as follows. And the corresponding data result was also updated in the manuscript with traced record.

(1) Revision for Figure 2 (B, D, E).

Purpose: To add standard deviation into Figure 2 (B, D, E), which illustrating the relationship of T1- or T2-weighted intensity and r1 or r2 relaxation-rate with iron concentration.

Methods:

The USPIO samples with gradient concentrations were contained in the 96-well plate to be conducted with MRI scanning. During data analysis, the two slices in the center at axial view of MRI images were chosen for further analysis. The T1-weighted or T2-weighted intensity, the T1 or T2 relaxation time, and $r1$ or $r2$ ($r1$ = 1/T1; $r2$ = 1/T2) relaxation rate were analyzed in voxel-based manner. These parameters for each voxel in the selected 2 slices were statistically analyzed and the mean and standard deviation were used to described the data distribution. Then, the mean values for T1-weighted and T2-weighted intensities under different iron concentrations were plotted along with standard deviation as shown in Figure 2(B). The mean values for $r1$ or $r2$ relaxation rate under different iron concentrations were further fitted by linear equation Eq. (3) in the manuscript which was also shown here. The curve fittings were also illustrated in Figure (D,E).

$$(1/T_i)_w = (1/T_i)_{wo} + r_i[\mathrm{Fe}],\ i = 1,2 \qquad \text{Equation (3)}$$

Revised Figure and Results:

The revised Figure 2 and the original data for figure plotting in the manuscript was placed here for reviewer's convenience.

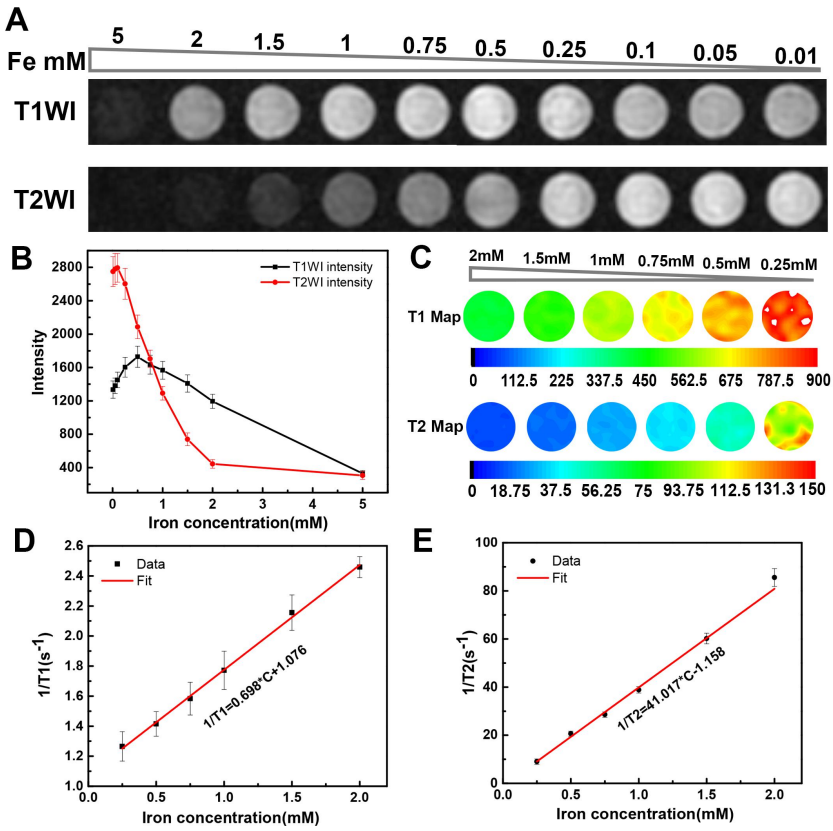

Figure 2. MRI properties of USPIO phantoms. (A) T1- and T2-weighted images of a series of 0.9% saline water solutions containing different concentrations of the USPIO probes as indicated by iron concentration. (B) Changes in the T1- and T2-weighted signal intensities according to iron concentration, with standard deviation also illustrated. (C) T1 and T2 map illustrated in pseudo color under different iron concentration. (D) Linear regression fitting of the longitudinal relaxation-rate (1/T1) and (E) transversal relaxation-rate (1/T2) data versus different iron concentrations (with standard deviation also illustrated) for extracting the longitudinal relaxivity (r_1) and transverse relaxivity (r_2), respectively.

Table C1 The T1-weighted T2-weighted intensities of USPIO samples with gradient iron concentrations which were illustrated in Figure 2(C).

Iron concentration (mM)	T1-W Intensity	T1-W intensity Standard Deviation	T2-W intensity	T2-W intensity Standard Deviation
0.01	1335	104.4402	2749.3	178.0192
0.05	1381.4	92.5607	2779.9	184.7643
0.1	1450.5	93.238	2793.3	171.6575
0.25	1603.1	118.6312	2603.3	185.9642
0.5	1729	126.8806	2087	141.6776
0.75	1633.9	113.4037	1703.4	103.9944
1	1566.6	106.004	1291.6	81.9133
1.5	1408.3	103.6123	738.996	76.3446
2	1194.1	83.9393	445.0242	51.2916
5	329.1895	37.0166	306.6633	46.4166

Table C2 The *r*1 and *r*2 relaxation rate of USPIO samples with gradient iron concentrations which were illustrated in Figure 2(D, E).

Iron concentration (mM)	r1	r1-STD	r2	r2-STD
0.25	1.2653	0.0978	9.0362	1.1133
0.5	1.415	0.0817	20.6223	1.0582
0.75	1.5834	0.1089	28.5981	1.0972
1	1.7718	0.1276	38.8605	1.3415
1.5	2.1556	0.1179	60.1715	2.2012
2	2.4586	0.0699	85.5549	3.6924

Note: r_1: longitudinal relaxivity; r_2: transverse relaxivity; STD: standard deviation.

(2) Revision for Figure 5.

Figure 5 in the manuscript illustrated the tumor-to-background ratio increasing rate over time. The data statistical error might be difficult to get because only maximumly two mice were used for averaging. Therefore, the data was re-analyzed for Figure 5 and the multiple intensity measurement was conducted to guarantee the reliability of the result.

Methods:

For each probe (U, UA, UG, UAG), there were two mice with orthotopic HCC prepared and received probe injection. The T1-weighted and T2-weighted images of these mice obtained at pre-injection, 12 h, 24 h and 32 h post-injection were further analyzed to calculate the tumor-to-background ratios and their increasing rate. For tumor intensity, the largest cross section of the tumor was delineated along the outer margin on T1-weighted or T2-weighted images and the mean intensities were recorded. Considering that the tumor might be not well recognized on T1-weighted images at pre-injection time point, the tumor was delineated on the pre-injection T2-weighted images and copied to T1-weighted images for intensity calculation. For liver parenchyma intensity, circular region of interest (ROI) with ~ 3mm^2 area in the liver lobe next to the tumor was drawn 2~3 times at different position and the average of the mean intensity in these ROIs were taken as the final background liver intensity. Then, the mean intensity of the tumor was divided by the averaged liver background intensity to obtain the tumor-to-background ratio. The ratio change rate was calculated by using tumor-to-background ratio at pre-injection as the denominator, which experience division by the difference of ratio at each time point post-injection and that of pre-injection. As there were two mice received each USPIO respectively, the ratio change rate at 12h, 24h and 32h for two mice was averaged. If image quality was not acceptable at some time point, the data at these time points were not included in the average process.

Revised Figure and Results:

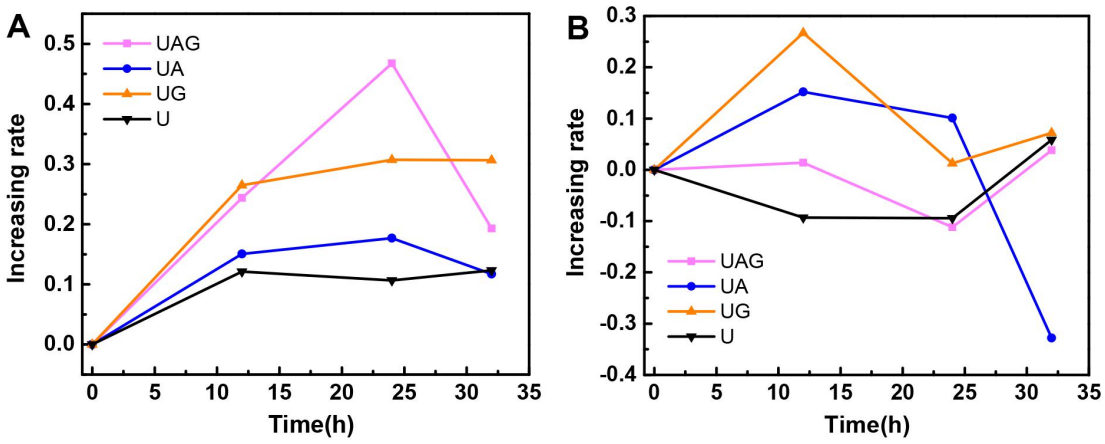

Figure 5. Time-dependent increasing rates of T1- and T2-weighted T/B signal ratios. Increasing rates of (A) T1- and (B) T2-weighted T/B signal ratio at different time points (12, 24, and 32 h) post-injection as compared with pre-injection (0 h) of the U, UA, UG, and UAG probes. Data points represent averaged rates from two mice if the image quality was acceptable.

Table C3 The T1-weighted MRI based tumor-to-background (T/B) signal ratio (TBSR) and its increasing rate measured in mice treated with different probes, including UAG, UG, UA and unlabeled USPIO.

UAG-T1WI image					
time	**T/B**	**RATE1**	**T/B**	**RATE2**	**Averaged RATE**

	ratio1		ratio2		
0	1.045	0.000	0.985	0.000	0.000
12	1.301	0.245	1.224	0.243	0.244
24	1.604	0.535	1.380	0.400	0.467
32	1.420	0.358	1.012	0.027	0.193
UG-T1WI image					
time	**T/B ratio1**	**RATE1**	**T/B ratio2**	**RATE2**	**Averaged RATE**
0	1.010	0.000			0.000
12	1.278	0.265			0.265
24	1.321	0.307			0.307
32	1.320	0.306			0.306
UA-T1WI image					
time	**T/B ratio1**	**RATE1**	**T/B ratio2**	**RATE2**	**Averaged RATE**
0	1.270	0.000	1.007	0.000	0.000
12	1.467	0.155	1.154	0.146	0.150
24	1.401	0.103	1.259	0.251	0.177
32	1.403	0.105	1.137	0.129	0.117
U-T1WI image					
time	**T/B ratio1**	**RATE1**	**T/B ratio2**	**RATE2**	**Averaged RATE**
0	1.027	0.000	0.953	0.000	0.000
12	1.158	0.127	1.063	0.115	0.121
24	1.197	0.165	0.999	0.048	0.107
32			1.070	0.123	0.123

Table C4 The T2-weighted MRI based tumor-to-background (T/B) signal ratio (TBSR) and its increasing rate measured in mice treated with different probes, including UAG, UG, UA and unlabeled USPIO.

UAG-T2WI image					
time	**T/B ratio1**	**RATE1**	**T/B ratio2**	**RATE2**	**Averaged RATE**
0	3.643	0.000	2.362	0.000	0.000
12	4.159	0.141	2.094	-0.113	0.014
24	3.443	-0.055	1.963	-0.169	-0.112
32	4.031	0.106	2.291	-0.030	0.038
UG-T2WI image					
time	**T/B ratio1**	**RATE1**	**T/B ratio2**	**RATE2**	**Averaged RATE**
0	3.625	0.000			0.000
12	4.593	0.267			0.267
24	3.671	0.013			0.013
32	3.886	0.072			0.072
UA-T2WI image					

time	T/B ratio1	RATE1	T/B ratio2	RATE2	Averaged RATE
0	3.784	0.000	2.961	0.000	0.000
12	4.420	0.168	3.363	0.136	0.152
24	4.147	0.096	3.275	0.106	0.101
32	2.355	-0.378	2.135	-0.279	-0.328
U-T2WI image					
time	**T/B ratio1**	**RATE1**	**T/B ratio2**	**RATE2**	**Averaged RATE**
0	3.127	0.000	1.782	0.000	0.000
12	2.652	-0.152	1.720	-0.034	-0.093
24	2.774	-0.113	1.647	-0.076	-0.094
32			1.886	0.059	0.059

We thank the reviewer for these valuable suggestions.

2. Images of immunohistochemical (IHC) staining are poor and aim, rationale and relevancy to MRI images are unclear. It is highly recommended to show IHC score even with semi-quantitative method.

Response:

We thank the reviewer for helping us strengthen the manuscript again!

During the experiment, it was a little difficult to locate the tumor sample embedded in the paraffin to have the identical orientation of MRI images. Therefore, in the current preliminary study, we could only speculate the probe targeting effects by relating the separate MRI-based and IHC-based results. Following the reviewer's suggestion, we learned and tried to analyze the IHC images by using semi-quantitative method as follows. The results were also added into the result part of "Histologic analysis".

Purpose: Analyze the IHC images based on semi-quantitative method and better connect the IHC results with the MRI findings to describe the targeting performance of different USPIO probes.

Method:

The semi-quantitative analysis was conducted on the histological images as shown in Figure 7 in the manuscript. The columns represented the tumor sections from mouse treated with U, UA, UG, UAG respectively. The rows labeled with "AFP", "GPC3" and "Prussian blue" were further analyzed by quantifying the ratio of DAB- or Prussian-blue-stained pixel among the pixels involved in the tumor region. The DAB-stained pixel ratio could reflect the AFP or GPC3

expression level and Prussian-blue-stained pixel ratio could reflect the targeting effects of iron-contained USPIO probes. The analysis steps were as follows.

Analysis for DAB-stained pixel ratio

Step1: Open the targeted AFP or GPC3 stained image in the Image J (Wayne Rasband and contributors, National Institutes of Health, USA. http://imagej.nih.gov/ij).

Step2: Subtract the image background with rolling ball radius set as 50 pixels.

Step3: Crop the image to only involve tumor region as much as possible.

Step4: Use IHC Profiler plugin to split the DAB-stained regions by choosing "Cytoplasmic Stained Image" mode. [*Varghese F, Bukhari AB, Malhotra R, De A (2014) IHC Profiler: An Open Source Plugin for the Quantitative Evaluation and Automated Scoring of Immunohistochemistry Images of Human Tissue Samples. PLoS ONE 9(5): e96801. doi:10.1371/journal.pone.0096801*] The DAB-stained ratio could be reported automatically.

Analysis for Prussian-blue-stained pixel ratio

Step1: Open the targeted Prussian-blue-stained image in the Image J (Wayne Rasband and contributors, National Institutes of Health, USA. http://imagej.nih.gov/ij).

Step2: Subtract the image background with rolling ball radius set as 50 pixels.

Step3: Split color channels into Red, Green and Blue. Choose the image in the Red channel and select the region automatically based on the threshold set as 210. Select the wrongly segmented region and fill them as background. Convert the retained segmented region into stained ROIs.

Step4: Delineate the tumor region and calculated the total pixel numbers Ntotal. Then calculate the pixel numbers located in the overlayed stained ROIs Nstained. The stained ratio is calculated as Ntotal/Nstained.

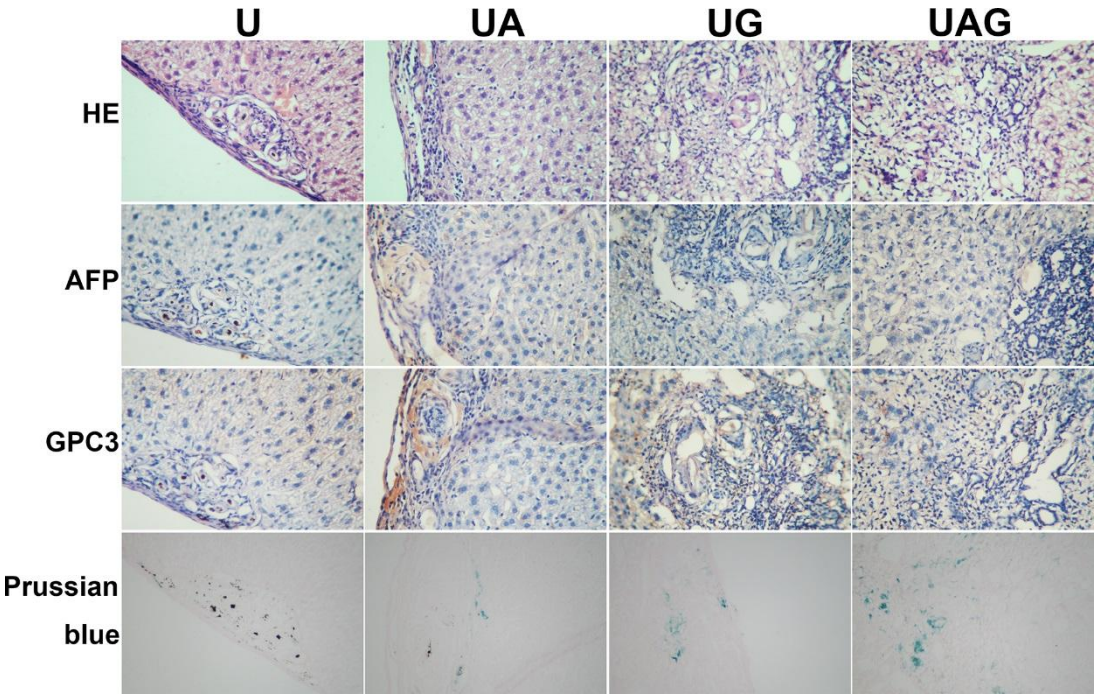

Figure 7. Histological results of HCC tumors treated with U, UA, UG and UAG probes.

Table C5 The targeted stained pixel ratio for AFP, GPC3 and iron in the U-, UA-, UG- and UAG-treated mice.

	U	**UA**	**UG**	**UAG**
AFP-stained ratio	25.96%	43.01%	25.49%	27.22%
GPC3-stained ratio	29.10%	38.19%	38.55%	39.72%
Prussian-blue-stained ratio	0.0040 (270328 px)	0.0051 (357189 px)	0.0124 (446022 px)	0.3848 (735700 px)

Table C5 summarized the results derived from the semi-quantitative methods above. Although it existed many aspects to improve the IHC sample staining and quantitative analysis in the current study, the semi-quantitative results might indicate that UAG-treated mice could have higher iron concentrated and targeting specificity in the tumor.

In the end, we sincerely thank the reviewer for the careful work and good suggestions.

2 Editorial Office's comments

1) **Science Editor:**

1 Scientific quality: The invited manuscript describes "Demonstration of a bi-specific T1 positive-contrast-enhanced magnetic resonance imaging molecular probe for hepatocellular carcinoma in an orthotopic mouse model". The topic is within the scope of the World Journal of Gastroenterology.

(1) Classification: Grade C;

(2) Summary of the Peer-Review Report: (00504218): This is a well-written paper containing interesting results. For the benefit of readers, however, a number of points need clarifying and certain statements require further justification. These are given below.

1. How many samples were analyzed in this study? Error bars should be shown in Figure 2 (B,C,D) and Figure 5 (A,B)

Response: DONE. Please refer to the response to the reviewer.

2. Images of immunohistochemical (IHC) staining are poor and aim, rationale and relevancy to MRI images are unclear. It is highly recommended to show IHC score even with semi-quantitative method;

Response: DONE. Please refer to the response to the reviewer.

(3) Format: There are 0 tables and 7 figures;

Response: Thank you editor! One table Table S1 was added in the supplementary information.

(4) References: A total of 50 references are cited, including 12 references published in the last 3 years;

Response: Thank you!

(5) Self-cited references: There is 1 self-cited reference;

Response: Thank you! The self-cited reference include the formal works characterizing the USPIO probes' physical properties and in vitro behaviors.

(6) References recommendations: The authors have cited proper references.

Response: Thank you!

2.Language evaluation: Classification: Grade B.

3 Academic norms and rules: The authors provided the Non-Native Speakers of English Editing Certificate. Institutional Animal Care and Use Committee Approval Form or Document. Biostatistics Review Certificate. Institutional Review Board Approval Form or Document.

4 Supplementary comments: This is an invited manuscript. The authors declare that there are no conflicts of interest regarding the publication of the paper. This study supported by CAMS Innovation Fund for Medical Sciences (CIFMS) (2016-I2M-1-001); PUMC Youth Fund (2017320010); Chinese Academy of Medical Sciences (CAMS) Research Fund (ZZ2016B01); and Beijing HopeRun Special Fund of Cancer Foundation of China (LC2016B15). The topic has not previously been published in the World Journal of Gastroenterology .

5 Issues raised: (1)The "Author Contributions" section is not detailed. Please provide the author contributions.

Response:

Thank you editor. The author contributions was added in the title page of the manuscript.

(2)The references should be updated.

Response: Dear editor, do you mean that the latest published articles in recent 3 years should be added?

6 Re-Review: Required.

Response: We thank the reviewer and editors for their hard works.

7 Recommendation: Conditional acceptance

2) Editorial Office Director:

3) Company Editor-in-Chief: I recommend the manuscript to be published in the World Journal of Gastrointestinal Oncology.

Response: Thank you for your recommendation. We will submit our revised manuscript to the WJGO.

We will look forward for your answers.

Best Regards,

Xiaohong Ma