Dear Dr. Jia-Ping Yan:

Thank you very much for your letter of February 15, 2019, with regard to the review of the manuscript entitled "**A seven- senescence associated gene signature predicts overall survival for Asian patients with hepatocellular carcinoma**" for consideration for publication in ***World Journal of Gastroenterology***. We are grateful to the reviewers' positive comments regarding our novel, significant and interesting results. We really appreciate the encouragement from both reviewers and the editor.

We have carefully considered each of the comments from all the reviewers and the editor, and explained every question. Below are our point-to-point responses, in which each comment from the reviewers is italicized and our responses are highlighted in blue. The revised texts in manuscript are highlighted in red.

Reviewer #1

Major

1. What is the clinical benefit of measuring the 7 gene signatures especially regarding cost-benefit.

Response: Thank you for your question.

To date, only Alpha-foetoprotein (AFP) was recommended as a first-line serological marker for HCC. AFP has been widely used in clinical practice both for HCC diagnosis. However, the value of serum AFP in predicting prognosis is limited [1]. As HCC is an extremely heterogeneous disease, it is necessary to seek for novel prognostic biomarkers to supervise patients at high risk for unfavorable clinical outcome.

In the present study, we construct the 7 gene signatures, whose level were detected from tissues, to evaluate the prognosis of postoperative patients. We can detected these seven genes using PCR, which is a specific, rapid and sensitive detective method. However, our findings were obtained based on tissue samples, which might limited their clinical application. Several published papers showed that MCM5 and CEP55 coded by two genes in our manuscript could be detected in serum. MCM5 was found to be abundant in the tumor secretome and was selected as CRC biomarker [2]. Meanwhile, CEP55 was also identified as a potential exosomal biomarker for head and neck squamous cell carcinoma [3]. In future, we plan to detect the serum levels of the 7 proteins that involved in this study and investigated their prognostic values.

References:

[1] H. Park, S.U. Kim, J.Y. Park, D.Y. Kim, S.H. Ahn, C.Y. Chon, K.H. Han, J. Seong, Clinical usefulness of double biomarkers AFP and PIVKA-II for subdividing prognostic groups in locally advanced hepatocellular carcinoma, Liver Int, 34 (2014) 313-321.

[2] M. de Wit, H. Kant, S.R. Piersma, T.V. Pham, S. Mongera, M.P. van Berkel, E. Boven, F. Ponten, G.A. Meijer, C.R. Jimenez, R.J. Fijneman, Colorectal cancer candidate biomarkers identified by tissue secretome proteome profiling, J Proteomics, 99 (2014) 26-39.

[3] F. Qadir, M.A. Aziz, C.P. Sari, H. Ma, H. Dai, X. Wang, D. Raithatha, L.G.L. Da Silva, M. Hussain, S.P. Poorkasreiy, I.L. Hutchison, A. Waseem, M.T. Teh, Transcriptome reprogramming by cancer exosomes: identification of novel molecular targets in matrix and immune modulation, Molecular cancer, 17 (2018) 97.

2. Do the authors have any data regarding the staging, treatment history and pathological information of the patients? If so, are there any relationship between the gene signature and these clinical information?

Response: Thank you for your question.

We have got data regarding the staging information, treatment history and pathological information of the patients in both two groups.

Stratified analysis showed that the risk score was associated with overall survival either in low or high pathological grades in TCGA cohorts. Due to the limited samples, we did not get statistically significance when analyzing treatment history. In GSE14520 dataset, we could not obtain the information of pathological grade and adjuvant treatment. Therefore, we did not perform the stratified analysis.

Our results showed the 7 SAGs have no specificity in predicting OS of HCC patients when considering tumor staging, treatment history and pathological, so we didn't show these data in our manuscript.

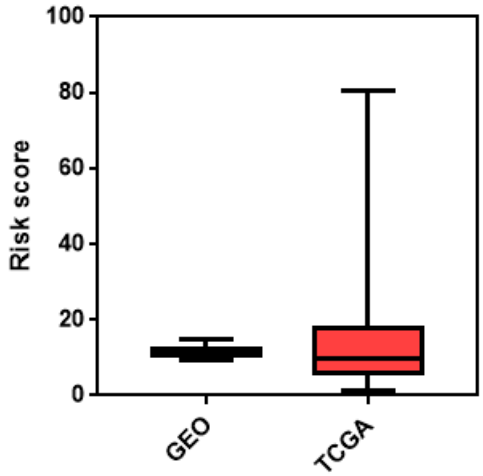
Stratified analysis of overall survival in GSE14520 and TCGA cohorts are shown in following Table:

Variables	**Discovery cohort**			**Validation cohort-Asian only**		
	High-risk / low-risk	**HR (95% CI)**	***P* value**	**High-risk / low-risk**	**HR (95% CI)**	***P* value**
TNM Stage						
I + II	45/57	1.64 (0.78-3.45)	0.178	49/64	6.39 (2.60-15.72)	<0.001*
III + IV	20/13	1.60 (0.70-3.63)	0.277	28/14	3.05 (1.32-7.04)	0.025*
Pathological grade						
G1+G2	*NA*	*NA*	*NA*	38/40	13.97 (5.69-34.28)	<0.001*
G3+G4	*NA*	*NA*	*NA*	41/38	3.13 (1.41-6.96)	0.010*
Adjuvant treatment						
Yes	*NA*	*NA*	*NA*	2/5	1.92 (010-35.53)	0.222
No	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*

3. Why do the authors set the cut-off value as the median of all patient's risk scores?

Response: Thank you for your advice.

TCGA dataset was performed on RNA-seq platform and GSE14520 was performed on gene-chip array platform. Based on the different platform and detecting methods, we can not pick up the same value as the cut-off value when analyzing the two groups. Moreover, the data vary widely in TCGA dataset, as shown in the following figure. Due to the characteristic of data, we set the cut-off value as the median, which was a widely used method cut-off method in previous studies.



Minor

"COMCLUSION "must be corrected to *"CONCLUSION"* in the abstract.

Response: Thank you for your advice, we have corrected the content in the abstract of our revised manuscript.

Reviewer #2

Major

The focus is interesting but the bridge to the clinical data set is poor. The information of liver cirrhosis level, child pugh score, AFP-L3 (%), background liver disease should be included in the tables and be compared.

Response: We totally agree with your comments.

Our findings were mainly obtained from on public datasets (TCGA and GSE14520). Unfortunately, we could not get the detailed information of Child-Pugh score and AFP-L3 (%), but only get the information of liver cirrhosis, AFP level and viral hepatitis infection status. Therefore, we performed subgroup analysis by stratifying patients according to the

above mentioned clinical variables. The results were shown in the following table.

In future, we plan to validate the prognostic value of the 7 SAGs in our own cohort and will perform stratified analysis according to your suggestions.

Table 2. Stratified analysis of overall survival in GSE14520 and TCGA cohorts

Variables	Discovery cohort			Validation cohort-Asian only		
	High-risk / low-risk	HR (95% CI)	*P* value	High-risk / low-risk	HR (95% CI)	*P* value
Cirrhosis						
Yes	70/69	1.93 (1.15-3.22)	0.012*	17/24	3.50 (0.66-18.47)	0.122
No	6/6	1.73 (0.10-30.65)	0.695	11/28	0.98 (0.19-5.00)	0.979
AFP (ng/ml)						
≤ 300	40/39	1.73 (0.77-3.87)	0.179	34/55	2.94 (1.01-8.57)	0.036*
> 300	34/34	2.11 (1.09-4.08)	0.025*	16/16	2.65 (0.60-11.66)	0.226
Viral hepatitis						
HBV	65/66	1.83 (1.05-3.19)	0.030*	41/34	3.05 (1.15-8.14)	0.042*
HCV	*NA*	*NA*	*NA*	19/12	0.50 (0.12-2.07)	0.222

Minor

A lot of typos. for e.g.,

Introduction outcome and adopt effective strategies

Methods Differential gene expression analysis The differential expression gene (DEG) analysis 3. The median was used as a cut off value for classification

Response: Thank you for your advice. We have corrected all typos in our revised manuscript according to your suggestions.

Reviewer #3

1. At introduction section, you have not listed a reference referring to the fact that AFP can predict the OS.

Response: Thank you for your advice.

We have added related references in the introduction section. Added sentences were highlighted as red in the revised manuscript as followings.

Traditional serum markers particularly alpha-fetoprotein (AFP) have been found to be prognostic in clinic [5-7]. However, they rely on significant tumor burden making their value limited in early stage tumors and elevated serum AFP was only observed in 60-70% of overall HCC patients [8].

References (in the revised manuscript):

[5] A.L. Komorowski, C.C. Hsu, K.D. Julka, B. Vasavada, C.C. Lin, C.C. Wang, C.L. Chen, AFP role in predicting recurrence of hepatocellular carcinoma after living donor liver transplantation in HCV patients, Neoplasma, 65 (2018) 455-460.

[6] A. Northen, T. Asendorf, P.D. Walson, M. Oellerich, Diagnostic value of alpha-1-fetoprotein (AFP) as a biomarker for hepatocellular carcinoma recurrence after liver transplantation, Clinical biochemistry, 52 (2018) 20-25.

[7] J.Y. Shen, C. Li, T.F. Wen, L.N. Yan, B. Li, W.T. Wang, J.Y. Yang, M.Q. Xu, Alpha fetoprotein changes predict hepatocellular carcinoma survival beyond the Milan criteria after hepatectomy, The Journal of surgical research, 209 (2017) 102-111.

[8] S. She, Y. Xiang, M. Yang, X. Ding, X. Liu, L. Ma, Q. Liu, B. Liu, Z. Lu, S. Li, Y. Liu, X. Ran, X. Xu, H. Hu, P. Hu, D. Zhang, H. Ren, Y. Yang, C-reactive protein is a biomarker of AFP-negative HBV-related hepatocellular carcinoma, International journal of oncology, 47 (2015) 543-554.

2.*At introduction, the statement: cellular senescence is considered a proliferating somatic cell that responds to…….etc, is not understandable.*

Response: Thank you for your advice. We have corrected the statement in the revised manuscript:

Cellular senescence could be induced prematurely by a variety of different types of stress and damage from both exogenous and endogenous sources. Persistent DNA damage is the most common cause of cellular senescence [9].

References (in the revised manuscript):

[9] T. Kuilman, C. Michaloglou, W.J. Mooi, D.S. Peeper, The essence of senescence, Genes & development, 24 (2010) 2463-2479.

3. *At introduction, no listed reference that indicate your statement about the role of RAS*

activation in senescence.

Response: Thank you for your question.

Senescence is known to occur in normal cells during the aging process as a result of telomere erosion, but it can also be induced prematurely by a variety of different types of acute stresses, e.g. UV irradiation and other DNA-damaging agents, hypoxia, toxins or overactive oncogenes like RAS. The latter is called oncogene-induced senescence (OIS) and is caused for instance by replicative stress and generation of reactive oxygen species (ROS) as a result of overstimulation of proliferation and cellular metabolism. This causes DNA damage that triggers the DNA damage response (DDR) leading to increased levels and activation of the tumor suppressor p53. p53 activates genetic programs involved in DNA repair, cell cycle arrest and senescence. According to your suggestion, we mentioned this point in brief in the introduction section and added related references in our revised manuscript. Considering our study did not mainly focus on OIS, we did not discuss too many detailed information.

The added information was highlighted as red in the revised manuscript:

For instance, amount of studies found that RAS activation could induced cellular senescence in many cancer cells. The replicative stress and generation of reactive oxygen species (ROS) caused by RAS activation triggers the DNA damage response (DDR) leading to activation of the p53 signaling pathway [12-14].

Reference (in our revised manuscript):

[12] A.J. Innes, J. Gil, IMR90 ER:RAS: A Cell Model of Oncogene-Induced Senescence, Methods in molecular biology (Clifton, N.J.), 1896 (2019) 83-92.

[13] L. Lau, G. David, Senescence Phenotypes Induced by Ras in Primary Cells, Methods in molecular biology (Clifton, N.J.), 1534 (2017) 17-30.

[14] L. Wang, R. Leite de Oliveira, C. Wang, J.M. Fernandes Neto, S. Mainardi, B. Evers, C. Lieftink, B. Morris, F. Jochems, L. Willemsen, R.L. Beijersbergen, R. Bernards, High-Throughput Functional Genetic and Compound Screens Identify Targets for Senescence Induction in Cancer, Cell reports, 21 (2017) 773-783.

4.At methods, both the survival analysis and the time dependent ROC curve are essentially statistical analysis and can be included with the statistical analysis.

Response: We totally agree with your suggestions. Both the survival analysis and the time dependent ROC curve are included with the statistical analysis in our revised manuscript as following.

Statistical analysis

Univariate and multivariate survival analysis were performed using the Cox proportional hazards regression model. Only variables with $P < 0.05$ in univariate analysis were incorporated into the multivariate Cox regression analysis. All tests were carried out using SPSS (version 22.0; Chicago, USA). Kaplan-Meier curves were generated using GraphPad Prism 7.0. Comparison between different subgroups was performed by the Log-Rank test. The median was used as a cut-off value to classify patients into high- and low- risk groups.

ROC curve is extended to evaluate biomarker's accuracy of discriminating binary outcomes [19]. Individuals who are disease-free earlier may develop the disease later and their markers' value may change from baseline during follow-up. Therefore, a time-dependent ROC curve analysis is more appropriate and outperforms the conventional method adopted for handling censored biomarker data. In this study, the time-dependent ROC curve analysis was performed by "survival ROC" package in R software (version 3.5.1). The prognostic performance was evaluated within 1-, 3- and 5-years to compare the predictive accuracy and sensitivity of different prognostic models.

The Chi-square test was carried out to discover the relationship between gene expression and clinical parameters. The difference of gene's expression in HCC patients of different features was analyzed by unpaired student's *t* test. *P* value less than 0.05 was considered statistically different. The statistical analysis was performed using IBM SPSS Statistics software program version 24.0 (IBM Corp, NY, USA).

5. *At discussion section, you have not discussed in a simple clear way the value and meaning of your results. For example, how to explain the meaning of the upregulation of the seven SAGs in HCC where you have mentioned that abrogation of senescence leads to development and aggressiveness of HCC. This is your explanation statement: (The potential explanation might be that due to the number of senescent cells increasing, the expression of 7- SAG signature is decreased, while patients with high expression indicate a higher proliferation rate and poorer OS); which is not satisfactory to explain the paradox.*

Response: Thank you for your comments. We have corrected this part in the discussion to make it more clearly. The revised part are highlighted as red in the revised manuscript.

Cellular senescence is primarily considered as a mechanism of tumor suppression that prevents cell transformation [20]. In liver tissues, chronic inflammation was an important stressor triggering senescence, leading to impaired hepatocyte regeneration [21]. The number of senescent hepatocytes was found to be increased during the progression of

chronic liver disease. Once hepatocytes breach the senescence barrier and they become immortalized [22]. Therefore, the dysfunction of senescence-related pathway in hepatocytes might affect patients' survival. In the present study, the seven senescence-associated genes were identified as lower expressed in the senescent cells and higher expressed in the HCC tissues. All seven genes played positive roles in the regulation of cell cycle. Silencing their expression caused cell cycle arrest and induced cellular senescence. According to our formula, the patients carrying lower expression of seven SAGs have a lower risk score, indicating these patients have a higher number of senescent cells and a better prognosis. Stratified results further suggested the 7- SAG signature was more applicable to the elderly HCC patients. The potential explanation might be that due to the number of senescent cells increasing, the expression of 7- SAG signature is decreased, while patients with high expression indicate a higher proliferation rate and poorer OS.

6. *At results, in the validation group, AFP was not a significant risk for HCC on multiple analyses.*

Response: Thank you for your question.

Sorry for our mistake in performing the Univariate/multivariate Cox regression in validation cohort. By re-analyzing the data, AFP was confirmed to be an independent risk factor for HCC patients in the validation cohort. The corrected data were listed in the revised manuscript.

Table 1. Univariate/multivariate Cox regression analysis of clinicopathologic factors associated with OS in GSE14520 and TCGA cohorts

Variables	Discovery cohort				Validation cohort-Asian only			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender (male/female)	1.36 (0.55-3.40)	0.507	–	–	0.89 (0.43-1.86)	0.755	–	–
Age (>60/≤60)	1.06 (0.57-2.00)	0.851	–	–	1.21 (0.66-2.24)	0.541	–	–
Cirrhosis (yes/no)	3.09 (0.76-12.65)	0.117	–	–	2.44 (1.56-3.85)	<0.001*	1.67 (1.04-2.70)	0.034*
AFP (>300/≤300 ng/ml)	2.30 (1.37-3.86)	0.002*	2.26 (1.38-3.69)	0.001*	3.70 (2.13-6.25)	<0.001*	2.13 (1.30-3.57)	0.003*
Risk score (High/Low)	1.93 (1.15-3.23)	0.012*	1.99 (1.19-3.34)	0.009*	5.91 (2.74-12.76)	<0.001*	4.22 (1.89-9.41)	<0.001*

Abbreviations: OS, overall survival; HR, hazard ratio; 95% CI, 95% confidence interval; AFP, Alpha-fetoprotein.

7. *You have not illuminated the relevance of your gene score whether it is related essentially to pathogenesis of HCC or otherwise it acts as a predictor and prognostic marker for HCC. The AUC of both AFP and your sophisticated SAG score are not greatly different. Thus the routine daily use of your gene score will not be applicable.*

Response: We totally agree with your comments.

As HCC is an extremely heterogeneous and poor prognostic disease, exploring biomarker for early diagnosis and predicting patients' response to novel treatment approaches is extremely urgent. AFP as a classic diagnostic biomarker for HCC showed limited value in predicting patients' prognosis. So far, there are many studies about seeking for biomarkers of HCC, but very few of them can be applied to the clinic. In the present study, we construct a risk score model consist of 7 genes, which were all played important roles during carcinogenesis. Our risk score was significantly associated with patients' OS. More importantly the risk score also showed a better predictive accuracy in predicting 1- and 3-year OS than AFP (1-year AUC=0.708 *vs* 0.606 and 3-year AUC=0.699 *vs* 0.568).

As you mentioned, the risk score was obtained based on tissue samples. Although we can detected these seven genes using PCR, which is a specific, rapid and sensitive detective method, the sample type might limited the possibility of routine daily use of our risk score. Recently, increasing studies discussed the possibility of developing diagnostic and prognostic biomarker using circulating cell-free nucleic acid. The expression and mutation of EZH2 has been reported to be detective in serum samples [1,2]. In future, we plan to detect the serum levels of the 7 genes that involved in this study and investigated their prognostic values. These works might provide the possibility for the routine daily use of our gene score.

Reference:

[1] Mehrotra M, Singh RR, Chen W, Huang RSP, Almohammedsalim AA, Barkoh BA, Simien CM, Hernandez M, Behrens C, Patel KP, Routbort MJ, Broaddus RR, Medeiros LJ, Wistuba II, Kopetz S, Luthra R. Study of Preanalytic and Analytic Variables for Clinical Next-Generation Sequencing of Circulating Cell-Free Nucleic Acid. J Mol Diagn. 2017;19(4):514-524.

[2] Rasmussen L, Christensen IJ, Herzog M, Micallef J, Nielsen HJ; Danish Collaborative Group on Early Detection of Colorectal Cancer. Circulating cell-free nucleosomes as biomarkers for early detection of colorectal cancer. Oncotarget. 2017;9(12):10247-10258.

8. Major language revision is warranted.

Response: We totally agree with your suggestion, and we have revised the language of our revised manuscript.

9. *Lastly, I wonder if the corresponding author can be more than one.*

Response: Thank you for your question. According to the format of the journal, we unanimously decided to keep the last one as corresponding author.