Peer Review File

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Reviewer A:

In this manuscript, the authors found that miR-126-3p contributes to sorafenib resistance in hepatocellular carcinoma via down-regulating SPRED1.

Major critiques:

1. What is the Clinicopathological characteristics of patients with HCC cancer according to miR-126-3p expression?

Response: We thank the reviewer's valuable suggestion. The data of HCC patients presented in the current study were download from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus. The Gene Expression Omnibus (GEO) is a public database that contains gene information including, high-throughput gene expression data, gene chips analyses, and microarray data. We searched the GEO database with the following keywords: "microRNA", "sorafenib" and "HCC", and identified the datasets: GSE56059 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56059). The dataset contains 20 hepatocellular carcinoma samples with partial clinical data. According to your constructive suggestion, we have added the original sample information of the 20 HCC patients download from GEO database, and we also organized the clinicopathological characteristics of the HCC patients in supplementary table 4.

Changes in the text: We added the original sample information of the 20 HCC patients in the supplementary materials named as "GSE56059 sample information. txt", and we also added the organized table of the sample information in the supplementary table 4.

2. Kaplan–Meier survival analysis of HCC patients with high or miR-126-3p should be supplied.

Response: Thanks for the reviewer's helpful comment. Since the original clinical data of the 20 HCC patients download from GEO database only contain the overall survival time but lack the survival status of the patient, our manuscript did not supply the Kaplan-Meier survival analysis with these incomplete data from GEO. However, to present as many as possible clinical informations, we have added the clinicopathological characteristics of the 20 HCC patients in the revised manuscript. In the following experiments, we will further enlarge the sample size to confirm the correlation of miR-126-3p with sorafenib resistance and survival prognosis in HCC patients.

3. Cell cycle assay should be added to verify cell proliferation results.

Response: Thank you very much for your kind suggestion. According to your suggestion, we used flow cytometry to detect cell cycle after the treatment of sorafenib, miR-126-3p inhibitor or combined therapy. The results demonstrated that sorafenib could markedly increase the percentage of cells in the G0/G1 phase of the cell cycle, while miR-126-3p inhibitor nearly had no effect on the cell cycle in MHCC97H cell. Moreover, combined miR-126-3p inhibitor with sorafenib also did not showed further enhancement in cell cycle arrest in MHCC97H cell (Supplementary Figure 3A). These results demonstrated that the synergistic enhancement effect of inhibition of miR-126-3p with sorafenib may not through regulation of cell cycle in HCC cells.

Changes in the text: We added the cell cycle results in the supplementary Figure 3A.

4. Cell cycle-related proteins (cyclin A/B/C/D/E, p21, p27, CDK2/4/6) should be detected in WB.

Response: We appreciate your comments very much. We are very sorry for that our laboratory doesn't have most of the antibodies you mentioned above. However, according to your suggestion, we detected the influence of miR-126-3p on the expression of cyclin D in HCC cells. The results demonstrated that miR-126-3p mimics or miR-126-3p inhibitor nearly had no effect on the expression in HepG2 or MHCC97H cells (Supplementary Figure 4A), and this was consistent with the result that miR-126-3p inhibitor nearly had no effect on the cell cycle in MHCC97H cell by the flow cytometry.

Changes in the text: We added the western blot result of cyclin D in the supplementary Figure 4A.

5. Expression of miR-126-3p in cancer tissues of 10 patients and their corresponding noncancerous mucosal tissues should be analyzed by IHC and western blot.

Response: Thank you very much for your comments. MicroRNAs (miRNAs) are short, conserved, non-coding RNAs that mainly regulate gene expression post-transcriptionally. Since miR-126-3p doesn’t have the function to translate into detectable peptides, the expression of miR-126-3p could not be detected by immunohistochemistry (IHC) and western blot assays. And the rational detection methods for the expression of miR-126-3p are gene chip assay and RT-PCR. In our current study, differentially miRNAs expression panels were analyzed between HCC patients with sorafenib non-response (progressive disease: PD) and those with

sorafenib response (partial response: PR or stable disease: SD). We analyzed the GEO dataset and detected the significant up-regulated expression of miR-126-3p in sorafenib resistant HCC patients, and the results showed that HCC cells with high expression of miR-126-3p exhibited increased resistance to sorafenib.

6. Does miR-126-3p affect death receptor-mediated apoptosis? Additional pro-apoptotic factors (eg. FasL, TNFa, TRAIL) should be added to test the effect of miR-126-3p on death receptor-mediated apoptosis.

Response: We appreciate the reviewer's comments very much. According to your suggestion, we added the flow cytometry to detect the effect of miR-126-3p on apoptosis in HCC cells. The results showed that miR-126-3p inhibitor could promoted cell apoptosis ratio from 6.22% to 10.38%. Previous studies have showed that sorafenib could induce cell apoptosis in HCC cells, thus we added sorafenib treatment as the additional pro-apoptotic factors. Our results further demonstrated that sorafenib could induce cell apoptosis of MHCC97H cell, while miR-126-3p inhibitor combined with sorafenib markedly induced cell apoptosis compared with either treatment alone (Supplementary Figure 3B). Taken together, these results showed that inhibition of miR-126-3p could enhance the anti-tumor effect of sorafenib against HCC cells by inducing cell apoptosis.

Changes in the text: We added the cell apoptosis results in the supplementary Figure 3B.

7. Does miR-126-3p affect the expression of other apoptosis-related mitochondrial proteins, for example, Bcl-2, Bcl-XL, Bax, Bim, Bad, IAPs?

Response: We thank the reviewer's valuable suggestion very much. According to your suggestion, we detected the influence of miR-126-3p on the expression of Bcl-2 (an anti-apoptosis protein) and Bax (a pro-apoptosis protein) in HCC cells by western blot assay. The results demonstrated that miR-126-3p mimics markedly up-regulated the expression of Bcl-2 and suppressed the expression of Bax in HepG2 cell, while miR-126-3p inhibitor significantly inhibited the expression of Bcl-2 and up-regulated the expression of Bax in MHCC97H cell (Supplementary Figure 4A). These results were consistent with the flow cytometry result that miR-126-3p could influence cell apoptosis in HCC cells.

Changes in the text: We added the western blot results of Bcl-2 and Bax in the supplementary Figure 4A.

8. What is the detailed mechanism of miR-126-3p regulating the cell proliferation, migration, and invasion of HCC cells? Does miR-126-3p affect AKT, Ras, Src and NF-kB signaling? EMT markers should be detected.

Response: Thanks for the reviewer's valuable suggestion. Compelling evidence has highlighted the notion that epithelial-mesenchymal transition (EMT) is an important mechanism of sorafenib resistance in advanced HCC. We also explored the effect of miR-126-3p on EMT markers (E-cadherin, N-cadherin and vimentin) by western blot assay, and we found that miR-126-3p mimics or miR-

126-3p inhibitor nearly had no effect on the EMT markers in HCC cells (Supplementary Figure 4B). Therefore, our results indicated that the mechanism of miR-126-3p with sorafenib resistance might not be related to EMT.

We carefully studied the related literatures about the mechanism of miR-126-3p. Previous studies have showed that IκBα is a potential target gene of miR-126-3p with a binding site in its 3'-UTR region in Hepatic Stellate cell, and they demonstrated that miR-126-3p could activate NF-κB signaling pathway through down-regulating IκBα protein expression.

In the present study, we demonstrated that HCC cells with high expression of miR-126-3p were more resistant to sorafenib treatment. To the best of our knowledge, this is the first time to report that miR-126-3p were correlated with sorafenib resistance in HCC. Then, bioinformatics analysis and the dual-luciferase reporter assay results showed that miR-126-3p could directly target SPRED1. By performing rescue assays upon the miR-126-3p/SPRED1 expression, our results demonstrated that miR-126-3p regulates sorafenib sensibility is dependent on regulating SPRED1 expression. SPRED1 is widely known as a negative regulator of the Ras/Raf/ERK signaling pathway. Previous study showed that SPRED1 could inhibit the ERK activation by forming a complex with Raf, and another study also demonstrated that SPRED1 inhibits the p-ERK levels through down-regulating Ras-GTP levels. Taken together, our results indicated that miR-126-3p promoted sorafenib resistance via targeting SPRED1 and activating the ERK signaling pathway.

Changes in the text: We added the western blot results of EMT markers in the supplementary Figure 4B.

Reviewer B:

1. **The legend of B and C is conversely in Figure 1.**

Response: Thank you very much for pointing out the mistake. We are sorry for this carelessness, and we have corrected the mistake in the revised manuscript. (see Page 24, line 508-511).

2. **If so, the expression of SPRED1 should be verified in HCC patients.**

Response: We appreciate the reviewer's valuable suggestion. In the present study, we analyzed the GEO dataset (GSE56059) and detected that miR-126-3p was significantly up-regulated in sorafenib resistant HCC patients. We confirmed that HCC cells with high expression of miR-126-3p were more resistant to sorafenib treatment. Furthermore, our current study revealed that SPRED1 served as a target of miR-126-3p and facilitated sorafenib sensibility in HCC cells.

A previous study has showed that the expression of SPRED1 was frequently decreased in HCC tissues comparing with adjacent non-tumorous tissues, and they also showed that reduced expression of SPRED1 was associated with tumor invasion and intrahepatic metastasis in HCC. In the present study, we mainly focus on the important role of SPRED1 with sorafenib sensibility, and we found that over-expression of SPRED1 markedly enhanced the anti-tumor effect of sorafenib against HCC. SPRED1 is widely known as a negative regulator of the Ras/Raf/MAPK signaling pathway, and the Ras/MAPK pathway is activated in 50-100% of HCC tissues and is correlated with a poor prognosis. Therefore, it is worthwhile for us to explore the more detail role and mechanism of SPRED1 in HCC and other tumors in the future. Thank you again for your helpful suggestions.