Response to reviewers

Dear Yuan Qi

Thank you very much for your letter and the reviewers' thought-provoking comments on our manuscript entitled "Dihydromyricetin-mediated inhibition of the Notch1 pathway induces apoptosis in QGY7701 and HepG2 hepatoma Cells" (Manuscript ID: 34562). We really benefit from your kind comments and we have checked the manuscript and revised it according to the comments. According to the order of reviews' suggestions, changes about our manuscript are listed. Besides, if you have any other suggestion, we would accept it with pleasure.

Reviewer 02860871 said: This article entitled "Dihydromyricetin-mediated inhibition of the Notch1 pathway induces apoptosis in QGY7701 and HepG2 hepatoma Cells" " by Lu et al is interesting in its field. The authors investigate whether DHM inhibits cell proliferation and promotes apoptosis by downregulating Notch1 expression. This article is well written with few typos inside. Here are some comments: **Major comments:** 1. Activation of Notch signaling is normally tightly controlled by direct interactions with ligand-expressing cells. It would be better if author also analyze the Notch ligand's activity in their cells or samples. 2. Please provide more result of the profile of Notch receptors and target genes in the samples. 3. There is an anomaly in patient number 2, which was Notch 1 is significantly higher in liver than in tumor. What is characteristic of this patient and what might be the possible explanation for this anomaly. 4. Author mentioned that DHM inhibits cell proliferation and promotes apoptosis by downregulating Notch1 expression. Please prove that by silencing the Notch1 genes may attenuate the inhibitory effects of DHM on the cells. **Minor comments:** 1. For figure 1a, please provide also the result of protein analysis for Hes1. 2. In figure 5, please provide the effectiveness of Notch1 siRNA. 3. Please provide the patients characteristics for all the 9 samples. The reviewer rated the manuscript as follows: **Classification Grade D (Fair), Language evaluation Grade B: Minor language polishing, Conclusion Major Revision.**

Answer:

1. Activation of Notch signaling is normally tightly controlled by direct interactions with ligand-expressing cells. It would be better if author also analyze the Notch ligand's activity in their cells or samples.

Response: Thanks for your suggestion; many studies have shown that activation of Notch signaling requires direct contact between Notch-expressing cells and cells expressing Notch receptors. However, our study focused on whether DHM regulates Notch1 and affects apoptosis in hepatocellular carcinoma (HCC) cells. Further attention to the notch ligand is a good direction in our future study.

2. Please provide more result of the profile of Notch receptors and target genes in the samples.

Response: Our team published an article in 2015 (Zhang et al. Oncotarget. 2015; 6(6): 3669-3679) and referred to the expression of Notch receptors (Notch1, 2, 3, 4) and downstream target genes. Due to the limited number of samples, we only found that Notch3 is correlated with occurrence of HCC. Many studies have shown that abnormal activation of Notch3 and Notch1 is correlated to HCC, Notch2 and Notch4 is not involved in the occurrence and development of HCC. In addition, we used DHM to treat HCC cells and we found that it only could downregulated Notch1 expression. Therefore, we consider furthering expanding the sample size to prove the association of Notch1 expression with hepatocellular carcinoma. We received a new sample of 64 patients; our results confirmed that Notch-1 overexpression is associated with HCC. So, we only showed Notch1 expression in the study.

3. There is an anomaly in patient number 2, which was Notch 1 is significantly higher in liver than in tumor. What is characteristic of this patient and what might be the possible explanation for this anomaly.

Response: Previous and our studies found that notch1 was highly expressed in most patients, and there was no significant difference in the expression level of notch1 in a few patients. In some patients, including number 2, Notch1 expression levels in HCC tissues were significantly lower than normal liver. At the same time we also consider this anomaly is due to individual differences between patients. The characteristic of patient number 2 is shown in the Response 7.

4. Author mentioned that DHM inhibits cell proliferation and promotes apoptosis by downregulating Notch1 expression. Please prove that by silencing the Notch1 genes may attenuate the inhibitory effects of DHM on the cells.

Response: Thank you for your advice. We found that DHM induced apoptosis of hepatocellular carcinoma cells by downregulating the expression of Notch1. In the experiment, the effect of DHM treatment group was similar to the Notch1-siRNA group. Furthermore, the ratio of Bcl2/Bax decreased the most in the Notch1-siRNA + DHM 100 μM group. Therefore, knockout of Notch1 may not attenuate DHM-induced apoptosis of hepatocellular carcinoma cells. We showed the above results in figure 5 and the data suggested that the effect of the Notch1-siRNA and DHM 100 μM combination on cell apoptosis was more obvious.

5. For figure 1a, please provide also the result of protein analysis for Hes1.

Response: This was done. Please see new version of the Figure 1.

6. In figure 5, please provide the effectiveness of Notch1 siRNA.

Response: This was done. Please see new version of the Figure 5.

7. Please provide the patients characteristics for all the 9 samples.

Response: The detailed characteristics for the 9 patients were shown in the following table.

Number	Age	Gender	Hepatitis B	AFP (μg/L)	Differentiation	Tumor size

	(years)					(cm)
NO.1	55	Female	+	134.27	Poor	≥ 5
NO.2	36	Male	+	20.37	High	< 5
NO.3	63	Male	+	102.72	Poor	≥ 5
NO.4	41	Male	+	217.22	High	< 5
NO.5	51	Male	-	210.36	Poor	≥ 5
NO.6	59	Female	+	120.37	Poor	≥ 5
NO.7	60	Male	-	19.35	Moderate+High	< 5
NO.8	44	Male	+	49.35	Poor+Moderate	≥ 5
NO.9	71	Male	+	216.91	Poor	≥ 5

Reviewer 00187828 said： The manuscript entitled "Dihydromyricetin-mediated inhibition of the Notch1 pathway induces apoptosis in QGY7701 and HepG2 hepatoma Cells" by Lu et al., was evaluated as follows; this is an interesting study in which authors demonstrated that DHM downregulates the expression of Notch1, Hes1 and Bcl-2 in QGY7701 and HepG2 cells. However, Bax was upregulated after the depletion of Notch1 via siRNA. Consequently down-regulation of Notch1 activates the mitochondrial apoptotic pathway. These results show that the anti-tumor activity of DHM in the QGY7701 and HepG2 hepatocarcinoma cell lines is partially exerted through inhibition of the Notch1 pathway. In summary, our experiments confirmed that Notch1 is involved in the development of HCC and may serve as a potential diagnostic marker for HCC and an indicator of malignancy. DHM inhibits cell proliferation and promotes apoptosis by down-regulating the expression of Notch1, indicating that it can be used as a candidate drug for the treatment of HCC. This is the first study to demonstrate that DHM inhibits cell proliferation and promotes. The reviewer rated the manuscript as follows: **Classification Grade A (Excellent), Language evaluation Grade B: Minor language polishing, Conclusion Accept.**

Response: We appreciate the favorable comments and the result of the evaluation of reviewer.

Reviewer 02861252 said： Good work. The reviewer rated the manuscript as follows: **Classification Grade B (Very good), Language evaluation Grade A: priority publishing, Conclusion Accept.**

Response: We appreciate the favorable comments and the result of the evaluation of reviewer.

We appreciate for Editors/Reviewers' warm work earnestly, and hope that this version will be accepted for publication.

Once again, thank you very much for your comments and suggestions.

Yours sincerely,

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