Author's response to reviews

Title: The Roles of Notch1 Expression in the Migration of Intrahepatic Cholangiocarcinoma

Authors:

Qi Zhou (hnzhouqi@163.com)
Ya-Feng Wang (eddy235@126.com)
Bao-Gang Peng (pengbaogang_2012@126.com)
Li-Jian Liang (lianglijian_2012@126.com)
Jia-Ping Li (jplli3s@medmail.com.cn)

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Author's response to reviews: see over
Dear Editor and Reviewers,

Thank you very much for giving us such important and constructive comments that need to be addressed. According to your advice, we have made major revisions to the original article seriously. In addition, American Journal Experts (AJE) has helped us to correct many errors in spelling, grammar, and word choice in our manuscript. The answers to the comments made by the reviewers and the changes made to the original manuscript are listed as follows:

Answer to the comments of Reviewer 1 (Dr. Hideo Baba):

1. Notch1 expression in human samples was indicated only by RT-PCR in figure 1. Author should demonstrated western blotting of Notch1.

Answer:
Thanks for your critical comment. As your advice, we already conducted additional experiments respect to western blotting of Notch1, cell proliferation assay with Notch1 knockdown and overexpression experiments, and determination of protein and transcript expression levels of Notch1 receptors in clinical samples as showed in Figure 1C, Figure 2, Figure 1C and Figure 5.

2. Author showed Notch1 and ICN expression level in cancer and noncancerous tissue in the same patient. However, major of noncancerous tissue should be hepatocytes, and normal biliary epithelial cells are not much.

Answer:
Thanks for your comment. In this study, the samples of ICC tissue and noncancerous tissue adjacent to the cancer lesion and normal liver tissues isolated from the same patient were enough to be performed reverse transcription-PCR, Western blotting analyses and immunohistochemical analysis. The primarily reason for our chose is that immunohistochemical result of Figure 1B was shown much better than other figures of immunohistochemical staining samples, but as your comments, normal biliary epithelial cells in the figure are not much in the figure. We also examined the expression of Notch1 in normal and ICC cells. As shown in Figure 1C, all cancer cell lines expressed high levels of Notch1 compared with normal biliary epithelial cells. The aberrant Notch1 expression in both ICC tissues and ICC cells suggests that increased Notch1 expression might be associated with tumor progression.

3. Cell proliferation assay should be performed in Notch1 overexpression and knockdown experiments.

Answer:
Thanks for your comment. We already added cell proliferation assay in Notch1 overexpression and knockdown experiments by BrdU incorporation analysis.

We overexpressed and knocked down Notch1 in ICC-9810 cells, which express moderate levels of Notch1. As shown in Figure 5, BrdU incorporation analysis indicated that Notch1 expression did not affect cell proliferation.

Noted in page1, line 1-3.

4. Generally, research manuscript should be made carefully. This manuscript has many crucial deficiencies. Figure 1 should be explained in the results section in detail. Figure 1 is inconsistent with the Legend of Figure 1. Figure 1.2 is not explained in the manuscript and figure legends at all. Figure 2A and 3A should be explained in detail (What do the lanes1-3 mean?). Figure 3 is inconsistent with the Legend of Figure 3.

Answer:
Thanks for your critical comment. We are very sorry for the some legends are inconsistent with figures in previous manuscript, we already carefully revised these mistake.

As your advice, Figure 1 explained in the results section in detail as follows:

Fig. 1. Notch1 is up-regulated in ICC tissues and ICC cell lines. (A) The expression of ICN (the intracellular domain of Notch1) is elevated in primary ICC tumors (T) compared with ICC tumor-adjacent tissues (N) examined by Western blotting. (B) The expression of Notch1 mRNA in each of the primary ICC tumor (T) and ICC noncancerous tissue (N) pairs from the same patient by reverse transcription-PCR. β-actin was used as a loading control. (C) The expression of ICN protein is elevated in ICC cell lines.

Other revised parts are noted in Figure legend 1-6

5. In the discussion section, lines 29-30 in page9 need the references.

Answer:

6. In the discussion section, lines 5-20 in page10 are only repeats of the results section.

Answer:
Thanks for your critical comment. We already carefully revised these words in the discussion section as follows:

In the present study, we found that Notch1 mRNA and ICN (the intracellular domain of Notch1) expression is higher in ICC tissue than in noncancerous tissue adjacent to the cancer lesions, and all cancer cell lines expressed high levels of ICN compared with normal biliary epithelial cells. Taken together, aberrant Notch1 expression in both ICC tissues and ICC cells suggests that increased Notch1 expression might be associated with tumor progression.

To elucidate the effects of Notch1 expression in ICC cells, separate overexpression and knockdown experiments were conducted in ICC-9810 cells. Notch1 cDNA was introduced into ICC-9810 cells, and Notch1 protein expression was successfully induced. Notch1 overexpression promoted migration and Rac1 activation in these cells. In contrast, the down-regulation of Notch1 inhibited the migration of ICC-9810 cells and resulted in dramatic decreases in Rac1 activity compared to control cells. Substantial evidence has indicated that increased Notch1 expression is accompanied by enhanced expression of α-SMA and Vimentin and loss of E-cadherin expression, which are hallmarks of EMT.

Answer to the comments of Reviewer 2 (Dr. Tung Yu Y Tsui):

1. The authors should provide more clinical data. Since the intrahepatic metastasis may differ from the lymphogenic metastasis, the authors have to provide the data of the expression of Notch in tumor and adjutant tissue in patients with or without early intrahepatic recurrence after surgery and with or without early lymph node metastasis.

Answer:

Thanks for your comment. We have provided the data of the expression of Notch in tumor and adjutant tissue in patients with or without early intrahepatic recurrence after surgery and with or without early lymph node metastasis in Material and Methods section as follow: Among the five cholangiocarcinoma patients, patients No. 1, 2, and 3 displayed infiltration of the surrounding tissue (invasion of the liver, portal vein, nerve, and pancreas), and patients No. 2 and 3 displayed regional lymph node metastases.

2. The expression levels of major receptors of Notch 1 should be determined in the clinical samples. The transcript and protein expression of targeted molecules should be quantified or semi-quantified.

Answer:

Thanks for your comment. As your advice, we have determined the expression of other Notch receptors (Notch2, Notch3, and Notch4) in ICC tissue and noncancerous tissue adjacent to the cancer lesions. As shown in Figure
2. Notch1 was found to be overexpressed in all five ICC cancer samples examined compared to normal adjacent tissue from the same patients, but the other receptors were not differentially expressed. We have noted that the proteins were detected by chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). The western blot data were quantified by measuring the intensity of the hybridization signals using an image analysis program (Fluor-ChemTM 8900, Alpha Inotech). The degree of Immunohistochemical staining was reviewed and independently scored by two observers based on the proportion of positively stained tumor cells and intensity of staining.

3. The conclusions in abstract is very ambitious which should be rephrased.
   Answer:
   Thanks for your critical comment. As your advice, we already rephrased the conclusions in abstract as follow:
   Thus, Notch1 may induce a migratory effect in ICC by causing an epithelial-mesenchymal transition and activating Racl and could serve as a novel diagnostic and therapeutic target in patients with ICC.

4. Quality of written English: Needs some language corrections before being published.
   Answer:
   Thanks for your critical comment. As your advice, we have made major revisions to the original article seriously. In addition, American Journal Experts (AJE) has helped us to correct many errors in spelling, grammar, and word choice in our manuscript.

The authors have nothing to disclose.

Thank you for your time and consideration.

Sincerely,

Jiaping Li, M.D., Ph. D.

Feb 16, 2013