Author’s response to reviews

Title: Elevated levels of circulating ITIH4 are associated with hepatocellular carcinoma with nonalcoholic fatty liver disease: From pig model to human study

Authors:
Naohiko Nakamura (n1982422@kuhp.kyoto-u.ac.jp)
Etsuro Hatano (shatano@hyo-med.ac.jp)
Kohta Iguchi (iguchik@kuhp.kyoto-u.ac.jp)
Motohiko Sato (fi920047@kuhp.kyoto-u.ac.jp)
Hiroaki Kawaguchi (hiroaki.kawaguchi1@gmail.com)
Iwao Ohtsu (ohtsu.iwao.fm@u.tsukuba.ac.jp)
Takaki Sakurai (sakurat@kuhp.kyoto-u.ac.jp)
Nobuhiro Aizawa (nobi23hiro@yahoo.co.jp)
Hiroko Iijima (hiroko-i@hyo-med.ac.jp)
Shuhei Nishiguchi (nishiguc@hyo-med.ac.jp)
Takuya Tomono (ctub2022@mail4.doshisha.ac.jp)
Yukihiro Okuda (yukihiro@kuhp.kyoto-u.ac.jp)
Seidai Wada (wadaseidai@gmail.com)
Satoru Seo (ruts@kuhp.kyoto-u.ac.jp)
Kojiro Taura (ktaura@kuhp.kyoto-u.ac.jp)
Shinji Uemoto (uemoto@kuhp.kyoto-u.ac.jp)
Masaya Ikegawa (mikegawa@mail.doshisha.ac.jp)

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Author’s response to reviews:

29-Mar-2019
Dear Editors: Dr. Xin Chen

BMC Cancer

Thank you very much for giving us the opportunity to revise our manuscript entitled “Elevated levels of circulating ITIH4 are associated with hepatocellular carcinoma with nonalcoholic fatty liver disease: From pig model to human study” by Nakamura et al. (BCAN-D-19-00994). We revised the manuscript according to the insightful comments and scientifically relevant suggestions from the Reviewers. First, we have detailed why ITIH4 could be used as a specific biomarker for NAFLD-related HCC. Second, we tried to quantify serum ITIH4 levels in human samples through ELISA. Third, we have provided new images of the 2D BN/SDS PAGE and western blotting results and added new graphs in the figures. Finally, the manuscript has been edited for grammar, spelling, vocabulary, and phrasing by native English speakers. We have highlighted the revised parts in the manuscript using red font in MS Word. We believe that the revised manuscript has benefitted substantially from the Reviewer’s comments. Thank you very much for your insightful comments and important suggestions.

Thank you for your consideration. We hope that our manuscript is now suitable for publication in your journal.

Sincerely,

Corresponding author:

Masaya Ikegawa MD, PhD

Department of Life and Medical Systems, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan
TEL: +81-774-65-6869, FAX: +81-774-65-6869,
E-mail address; mikegawa@mail.doshisha.ac.jp

Etsuro Hatano MD, PhD

1-1 Mukogowacho, Nishinomiya, Hyogo, 663-8501, Japan
Department of Surgery, Hyogo College of Medicine, Hyogo, Japan
TEL: +81-798-45-6582, Fax number: +81-798-45-6581,
E-mail address; shatano@hyo-med.ac.jp

Point-by-point responses to Reviewer comments:

Reviewer reports:
NAFLD is a widely spread disease in western countries and is rapidly growing in developing countries. Since NAFLD can evolve to HCC and there is no any evident symptoms at its early stage, effective diagnosis will be the key for early therapeutic intervention. In this study, Nakamura et al established a novel pig model to mimic human HCC with NAFLD background. Then they examined the serum marker and found that ITIH4, a protein secreted by hepatocytes, was elevated significantly in the swine serum. In concert, ITIH4 was also increased in HCC patients with NAFLD but not in the patients with hepatosteatosis, NASH or hepatic virus infections. These results indicate that ITIH4 holds the potential to be used as a early diagnosis biomarker for those population with NAFLD. This paper provides a new biomarker for diagnosis of HCC based on the data from a pig HCC model, which is convenient for multiple collections of serum. Both the finding and animal model will be helpful for the researchers or clinicians in this field. However, some concerns about the data or writing needs to be clarified before it is accepted for publication.

Major concerns:

1. The authors used many times in the figure legends and results section, but they failed to define what the control group refers to. Does that refer to the pigs injected with DEN but not fed with HFD? The authors should clarity it.

<Response>

Thank you very much for your insightful comments. The pig in the control group was fed a normal diet but not injected with DEN. We are sorry for the confusion. We have revised the text in the methods section (line 9, page 10) as follows: “On the contrary, a pig that was fed a normal diet and was not administered DEN was used as the control.” We also provided additional descriptions in the figure legends: “The NAFLD group; HFD feeding with DEN injection. The control; normal diet feeding without DEN injection” (line 9, page 42).

2. For the serum collection and MS-SPEC analysis, how much volume of blood was collected and how much serum/protein was used for HPLC-MS analysis? The details for this experiment will be critical for other group to repeat or to use it as reference.

<Response>
Thank you very much for your thoughtful advice. We have reported the amount of serum collected from the animals in the methods section (line 16, page 11) as follows: “10 ml of serum was separated by centrifugation at 3500 rpm for 10 min and stored at –80°C immediately.” The volume of the samples that was applied to the BN-PAGE gels has also been reported in the methods section (line 7, 16, page 13) as follows: “First, 2 µl of serum was placed on a centrifugal filter column (Amicon Ultra 3K; Millipore, Billerica, MA, USA) with 500 µl of blue native buffer.” “In the first-dimensional BN-PAGE, 12 µl of sample was applied per lane to a 4 to 15% gradient gel”.

3. The data in Figure 3 showed that ITIH4 increased rapidly from 36-60 weeks and the HCCs were formed in all the pigs they used at 60 weeks. This reviewer is wondering when actually those HCC started to originate during 36-60 weeks assuming ITIH4 is reliable marker for HCC formation? If ITIH3 starts to rise around the HCC formation, then it will be an excellent marker for early diagnosis of HCC.

< Response>

Thank you very much for your insightful comments and valuable suggestions. Although we sacrificed the animals at 60 weeks, we confirmed the development of liver tumors in the two pigs of the NAFLD group at 48 and 52 weeks, respectively, when gross observations of the whole liver were performed through laparotomy. Tumor development was not macroscopically observed through laparotomy at 36 weeks. Therefore, we believe that serum ITIH4 elevation could correspond with HCC formation.

Minor questions

Figure 1. The authors may use multiple columns instead of single tandem column to quantify the pathological scores, since the former will easier for readers to interpret and compare.

< Response>

We have revised Figure 1 according to your kind suggestion.

Figure 3C. The replicate numbers and error bars should be shown or indicated in the graph or figure legend.

< Response>

We have provided error bars and replicate numbers in Figure 3C and its legend (line 17, page 43).
Siu Tim Cheung (Reviewer 2): Nakamura et al. reported the study entitled "Elevated levels of circulating ITIH4 are associated with hepatocellular carcinoma with nonalcoholic fatty liver disease: From pig model to human study”.

Major issues:

1. Elaborate and justify the use of 2 and 1 animals for high fat diet and control diet respectively.

<Response>

We respect your thoughtful suggestions. As you pointed out, the current study lacks statistical strength due to the small sample size, however, detailed chronological comparison on the same experimental set lead to the discovery of a serum biomarker for NAFLD-related HCC. That is to say, an advantageous aspect of pig as an animal model is that genetically matched, serial serum samples can be analyzed using the same experimental set.

As for experimental protocol, we had established an NAFLD-HCC mouse model and this is the reason that we can generate swine NAFLD-related HCC model with a limited number of animals. In the mouse model, NAFLD-related HCC could be successfully induced by a high fat diet administered along with DEN injection, but not by administering a high fat diet or DEN injection alone. In addition, the hepatic expression of ITIH4 in NAFLD-HCC mice was significantly higher than that in control animals or in mice with NAFLD but not HCC. We showed these results in the supplementary materials (unpublished data). We have described the limitations of this study in the discussion section.

2. The 2D BN/SDS PAGE (Figure 3A) and western blot (Figure 3B; 7A) have quality concerns. Provide alternative images.

<Response>

We have provided new images of 2D BN/SDS PAGE and western blotting results.

3. Figure 7A western blot: Clarify if there is loading control.

<Response>

Thank you very much for your valuable suggestion. Since we performed western blotting using a defined amount of each human serum (2 μl) with 10 μl of sample buffer, total protein input for each sample must be exactly the same amount. For example, we have calculated input protein amount in each sample by Bradford method (SS: n=10, NAFLD-HCC: n= 10, NASH: n=10) to find out that around 0.5 μg of protein per one sample was loaded in each lane.
4. Serum ITIH4 (Figure 6) levels were measured by western blot / densitometry. Serum levels should be examined by ELISA for quantitative measurement.

< Response>

Thank you very much for your advice. We have purchased ELISA kit (DuoSet® ELISA: DY8157-05) for the quantitative measurement of serum ITIH4 levels in human samples and unfortunately, it did not work for unknown reason. It is true that the quantitative measurement of serum ITIH4 levels is essential for supporting our hypothesis, however, this time we could not substitute by ELISA for the moment. By the next study, we will find a reasonably working immunoassay to detect ITIH4 level in human samples as well as current analysis.

5. Clarify if Figure 3B ITIH4 is western blot for the 120kDa or 35kDa protein. Should evaluate both protein size. Figure 7A showed both 120kDa and 35kDa ITIH4 protein.

< Response>

The blot in Figure 3B shows a 120 kDa ITIH4 band. In the western blot using human serum, the 35kDa ITIH4 band could be detected (Figure 7A). However, we did not detect the 35 kDa ITIH4 band in the western blot using pig serum. Although it is unclear why 35 kDa ITIH4 was undetectable in pig serum, one possibility is the different antibodies used in each western blotting validation experiment for pig and human may have affected the results.

6. There are reports on the evaluation of serum ITIH4. Authors should discuss the similarity and uniqueness between the current study and the literature:


< Response>

Thank you very much for your valuable comments. In the above-mentioned reports, decreased serum ITIH4 levels were seen in HCC patients with hepatitis virus-related cirrhosis. Our findings also indicated that serum ITIH4 levels were lower in virus-related HCC patients. Thus, higher ITIH4 expression appears to be associated with etiology-dependent, especially obesity- or metabolic syndrome-dependent, carcinogenesis. We have discussed these reports and have provided additional information in the discussion section (line 17, page 28).